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THE ROLE OF CARBOXYLIC ACID GROUPS IN THE ACTION OF GLUCOAMYLASE I

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1. Introduction

Carboxylic acid groups have been shown to be present in the active sites of many enzymes, including pepsin [1], β-D-glucosidase [2], lysozyme [3] and carboxypeptidase A [4], and their presence has been suggested in many other cases [5]. Often the first indication that a carboxylic acid group is involved at the active site of an enzyme is obtained from the pH-rate profile, an ionizable group with pK_a about 3–5 being frequently assumed to be a carboxyl. Conclusions based on pH-studies, however, must always be confirmed in other ways. A variety of methods have been used for the chemical modification of carboxyl groups in proteins and these can be valuable in structure-function correlations. For example, p-bromophenacyl bromide [6] and 1-diazo-6-phenyl-3-toluenesulphonamidobutan-2-one [7] inactivate pepsin by forming esters with the side-chain carboxyl of an essential aspartyl residue, and conduritol B-epoxide inactivates β-D-glucosidase [8] by reaction with a carboxyl at its active site.

Hoare and Koshland [9] developed a method for the modification of carboxyl groups in proteins, which involves the reaction of glycine methyl ester in the presence of a water soluble carbodiimide. We have studied the effect of this reaction on the activity of glucoamylase I, an enzyme whose pH-rate profile had suggested the possibility that a carboxyl group might be implicated in the enzymic process.

2. Materials and methods

Crude glucoamylase from *Aspergillus niger* (Agidex 3000) was purified by chromatography on DEAE-cellulose as previously reported [10]. Glycine methyl ester hydrochloride was obtained from Koch-Light Laboratories and was virtually homogeneous on thin-layer chromatography. *N*-Ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was obtained from Fluka AG.

The reaction procedure was essentially that described by Hoare and Koshland [9]. In a typical experiment, glucoamylase I (5.7 mg) was incubated in water with glycine methyl ester hydrochloride (0.7 M) and EDC (75 mM) at 25°. The pH was maintained at 4.75 by addition of 1 M hydrochloric acid using a Radiometer pH-stat. The total initial volume was 2.0 ml. Aliquots (100 μl) were withdrawn at appropriate intervals and added to 0.2 M acetate buffer, pH 4.5 (2.0 ml) to quench the reaction. These solutions were then stored at 4°. In control experiments it was demonstrated that no enzymic activity was lost in the presence of 0.2 M acetate or if EDC was excluded from the reaction mixture. In addition both glycine methyl ester and EDC were shown not to interfere with the glucoamylase assay. Glucoamylase activities were determined from the release of glucose from starch (1% in 0.2 M acetate buffer, pH 4.5), liberated glucose being analyzed by the glucose oxidase method of Dahlqvist [11].

In parallel experiments to obtain samples for amino acid analysis, 500 μl aliquots were removed at intervals and chromatographed on Bio-Gel P10 equilibrated with 0.2 M acetate buffer pH 4.5. Fraction containing protein were pooled. Amino acid analyses were carried out using a Technicon Autoanalyzer after hydrolysis of samples in 6 M hydrochloric acid in evacuated sealed tubes at 107° for 24 hr.

Intercepts in the semi-log plots shown below were calculated by a least-squares procedure.

3. Results and discussion

Fig. 1 is a plot of glucoamylase activity remaining *vs.* time of reaction, the former being on a log scale. Clearly the reaction causes considerable loss of enzymic activity. Indeed after 1 hr no activity could be detected. Two processes appear to be involved, since the points lie on two straight lines. Calculation of the intercept obtained by extrapolation of the second line, corresponding to a slower reaction, gives a value of 79.3% indicating that about 20% of the activity is lost rapidly, the remainder being destroyed more slowly by a first order process.

Reactions were then carried out in the absence and in the presence of maltose, a substrate for glucoamylase, in order to determine whether a substrate would protect the enzyme from inactivation. The results are illustrated in fig. 2. In the absence of maltose the loss of activity is rapid, the initial loss of 20% again being clear. In the presence of maltose the rapid initial loss of activity still occurs, but the subsequent process is almost completely suppressed. Clearly the initial loss of activity is not prevented by the substrate which does protect the enzyme from the major loss. It is probable, therefore, that the initial loss of up to 20% of the activity is caused by a non-specific process, not at the active site.

A further experiment was carried out in which the enzyme was first treated with the reagents in the presence of maltose for 2 hr. The product obtained possessed 66.3% of the original activity. It was then chromatographed on a column of Bio-Gel P-10 to remove the maltose and subsequently retreated with the reagents. Fig. 3 shows the plot of loss of activity with time. The activity was lost by a single first order process, no initial rapid loss being apparent. Prolonged reaction totally destroyed the activity.

Further light is thrown on the process by amino acid analysis of the products. The native enzyme contains 47 residues of glycine per molecular weight of proteins of 61 500 [10, 12, 13]. The reaction introduces one extra glycine residue per carboxylic acid group reacting. The table shows that the reaction in the absence of maltose leads to an incorporation of about 38 more glycine residues. Furthermore, about 36 of these are present after only 4.5 min. Prolonged

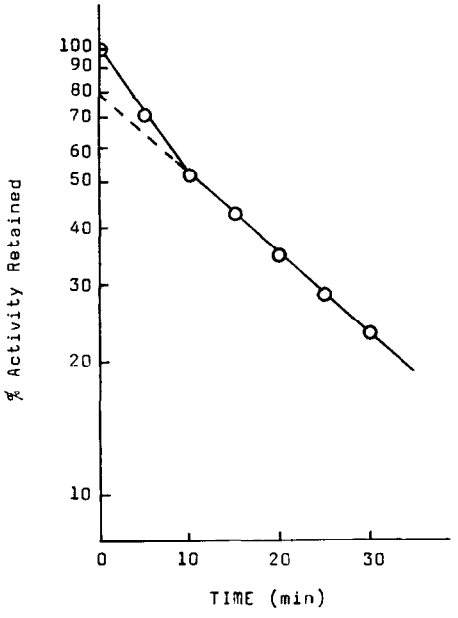

Fig. 1. Loss of activity of glucoamylase I on treatment with glycine methyl ester and *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide. The intercept shown is calculated as 79.3%.

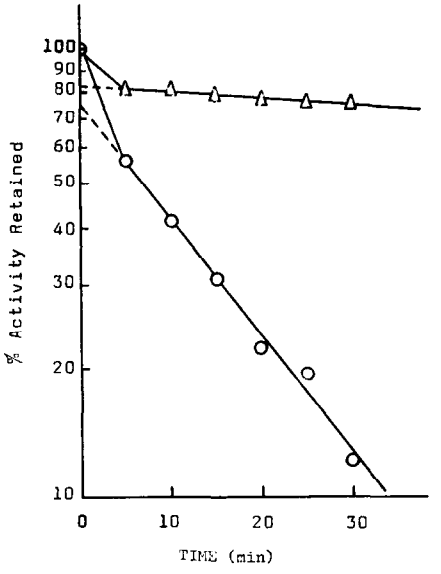

Fig. 2. Loss of activity of glycoamylase I on treatment with glycine methyl ester and *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide in the absence (circles) and the presence (triangles) of maltose. The intercepts shown are calculated to be: maltose present, 82.6%; maltose absent, 77.4%.

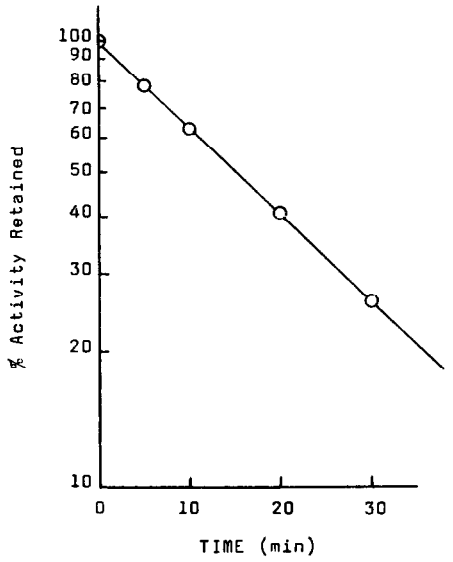

Fig. 3. Loss of activity of glycoamylase I (previously treated with glycine methyl ester and *N*-ethyl-*N'*-(3-dimethylamino-propyl) carbodiimide in the presence of maltose) on treatment with the same reagents in the absence of maltose.

Table 1

Total glycine residues per 61,500 molecular weight of protein*, after reaction of glucoamylase I with glycine methyl ester and *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide.

Time of reaction (min)	No. of glycine residues	
	Maltose absent	Maltose present
4.5	83.1	n.d.
60.0	85.5	82.7
120.0	84.5	83.3

* The molecular weight of the *protein* in native glycoamylase I is quoted as 61,500 [10, 13]. These figures correspond to this value in that they have been calculated on the basis of protein molecular weight of 58,830 ignoring glycine.

n.d. –not determined.

reaction leads to the introduction of only 2–3 residues more. Presumably these are the residues whose modification causes the major loss of activity. This is confirmed by the results of the reaction in the presence of maltose where the total number of glycine residues incorporated is 2–3 residues smaller. It is the reaction of these few residues which is prevented by the presence of substrate.

In view of these results we conclude that probably 2–3 carboxylic acid groups are present at the active site of glucoamylase I, and modification of these causes loss of enzymic activity. Furthermore, about 36 carboxylic acid groups appear to be exposed generally over the surface of the molecule, and it is the reaction of these residues which causes the initial rapid loss of about 20% of the activity. A loss of activity of this nature brought about by such a process, is not unexpected since the reaction results in the destruction of one negative charge per residue and is, therefore, liable to produce a serious conformational change.

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