Carbohydrate Research, 49 (1976) 361–370

TRYPTOPHANYL AND CARBOXYLIC ACID RESIDUES IN THE ACTIVE CENTRE OF GLUCOAMYLASE I FROM *Aspergillus niger**

MICHAEL E. JOLLEY AND CHARLES J. GRAY

Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (Great Britain)

(Received December 1st, 1975; accepted for publication, January 8th, 1976)

ABSTRACT

The pH-dependence of the photo-oxidation of L-tryptophan, in the presence of Rose Bengal and Methylene Blue, has been investigated. True, initial rate constants were determined in order to circumvent errors due to secondary processes. Photo-oxidation of glycoamylase I from *A. niger* in the presence of Methylene Blue or Rose Bengal resulted in a pH-dependent loss of enzymic activity, which was analogous to the destruction of free L-tryptophan during photo-oxidation. The loss of enzymic activity was closely associated with the destruction of tryptophan residues in the enzyme. Significant protection of both enzymic activity and tryptophanyl residues in the enzyme molecule was achieved by performing the photo-oxidation in the presence of maltose, which is a substrate for the enzyme. The tryptophanyl residues of glucoamylase I, which had been inactivated by reaction of its carboxylic acid residues with glycine methyl ester in the presence of a water-soluble carbodi-imide, were also substantially protected by maltose. It is concluded that the active centre of glucoamylase I is a cleft lined with tryptophanyl residues that participate in the binding of the substrate. One or more carboxylic acid residues are involved in bond cleavage.

INTRODUCTION

Glucoamylase I [(1→4)-α-D-glucan glucohydrolase, EC 3.2.1.3] catalyses the hydrolysis of starch, producing D-glucose as the main product. In its mode of action, tryptophanyl and carboxylic acid residues have been implicated[1,2]. The activity of the enzyme may be abolished by reaction of its carboxylic acid residues with glycine methyl ester in the presence of ethyl (3-dimethylaminopropyl)carbodi-imide[2] (EDC), or by photo-oxidation in the presence of Methylene Blue[1]. In the latter process, the enzymic activity is lost by a pH-dependent process. Tryptophan is the only amino acid destroyed[1].

We have suggested[1] that the pH-dependence of this process is indicative of a conformational change in the enzyme[1]. Our conclusions were based on a report that

*Dedicated to the memory of Professor Edward J. Bourne.

the photo-oxidation of free tryptophan in the presence of Rose Bengal is not pH-dependent, as determined by oxygen uptake[3]. We have therefore investigated this process further by determining tryptophan chemically[4], and now report the results of these studies.

MATERIALS AND METHODS

Glucoamylase I from *A. niger* (Agidex 3000) was obtained pure by chromatography on DEAE-cellulose as previously described[1,5,6].

Photo-oxidations were performed in tubes (76 × 12 mm) placed in a water-cooled jacket. Samples, presaturated in the dark with oxygen, were illuminated by a 240-watt Phillips "Photoflood" lamp at a distance of 15 cm. Water-saturated oxygen was bubbled through the solution during illumination to ensure complete saturation. Aliquots were withdrawn at intervals and stored in light-proof tubes at 4° for assay. Initial, first-order rate constants were determined from the initial slope of the plot of the logarithm of the fraction of tryptophan (or enzyme activity) remaining against time of photo-oxidation.

Glucoamylase activities were determined by the release of D-glucose from starch (1% in 0.2M acetate buffer, pH 4.5), liberated D-glucose being determined by the D-glucose oxidase method of Dahlqvist[7].

Tryptophan was determined by the method of Spies and Chambers[4].

RESULTS

Fig. 1 shows the pH profile for the initial rate of destruction of tryptophan during photo-oxidation in the presence of Rose Bengal. The initial rate constant increased slightly between pH 4.0 and 8.0. Above pH 8.0, the initial rate constant increased rapidly.

The results of a similar experiment, performed in the presence of Methylene Blue, are shown in Fig. 2. In this case, the initial rate constant was markedly pH dependent throughout the whole pH-range employed. Below pH 4.0, no tryptophan was destroyed. Above pH 8.0, there was a very rapid loss of tryptophan.

Fig. 3 shows the pH-dependence of the initial rate of loss of tryptophan in the glucoamylase I, compared to those for the loss of enzymic activity[1] and destruction of free tryptophan (Fig. 2), during photo-oxidation in the presence of Methylene Blue.

Fig. 4 shows the effect on the enzymic activity of glucoamylase I of photo-oxidation in the presence of Rose Bengal at pH 4.5 and 9.0. The rate of loss of activity at pH 4.5 was signiffcant (42.5%) when compared to that at pH 9.0. The rate of destruction of free tryptophan at pH 4.5 was 41.2% of that at pH 9.0 (see Fig. 1).

As can be seen from Figs. 1 and 2, the rate of destruction of free tryptophan at pH 4.5, the optimal pH for glucoamylase I, during photo-oxidation in the presence of Rose Bengal, is far greater than that when the same concentration of Methylene Blue

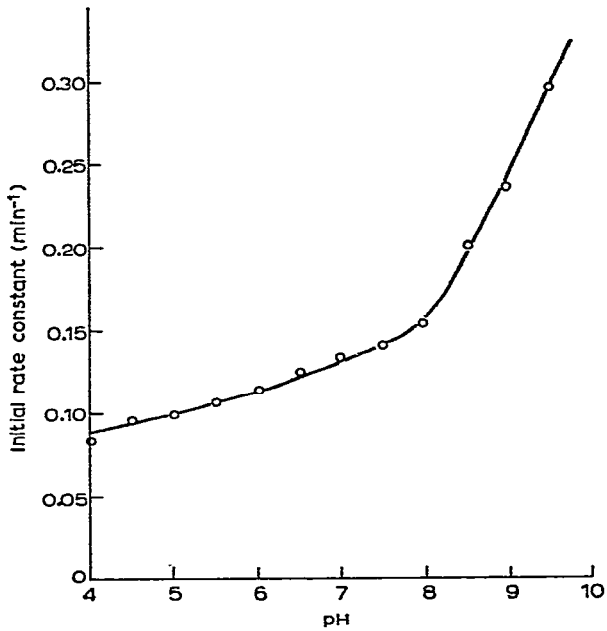

Fig. 1. The pH-dependence of the initial first-order rate constant for the destruction of L-tryptophan during photo-oxidation in the presence of Rose Bengal. Solutions, at each pH value, initially contained tryptophan (110 μg) and Rose Bengal (10 μg) in a total volume of 1.2 ml, illuminated at 13.0°.

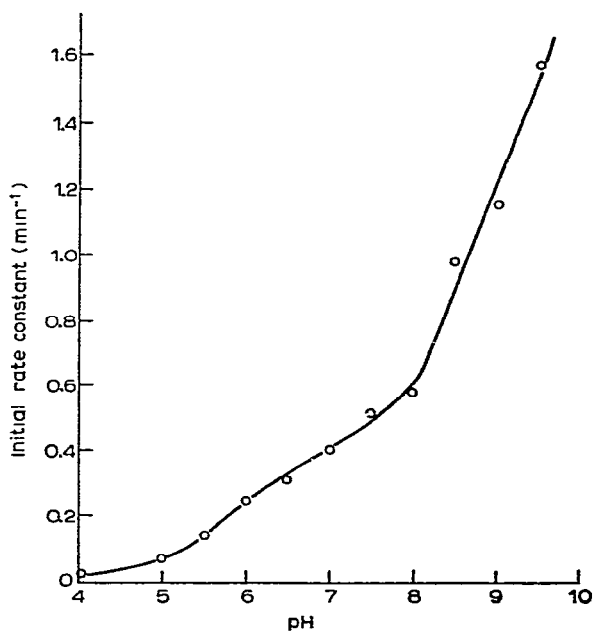

Fig. 2. The pH-dependence of the initial first-order rate constant for the destruction of L-tryptophan during photo-oxidation in the presence of Methylene Blue. Solutions, at each pH value, initially contained tryptophan (110 μg) and Methylene Blue (10 μg) in a total volume of 1.2 ml, illuminated at 13.0°.

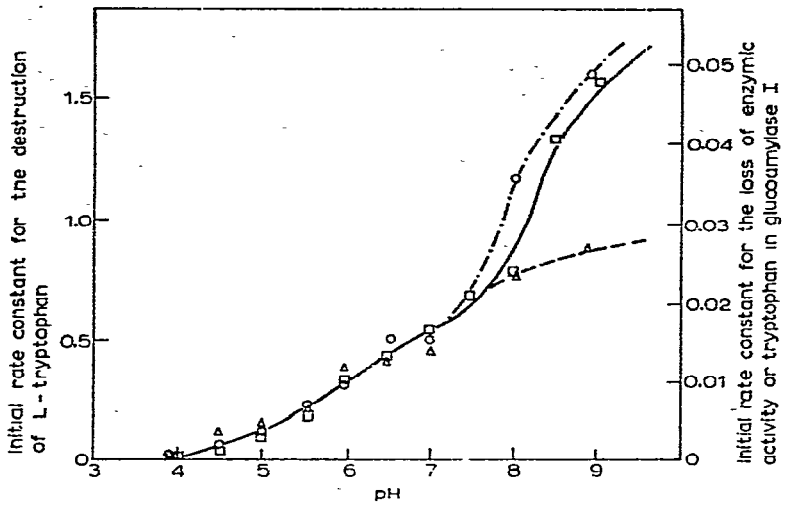

Fig. 3. The pH-profiles for the initial rate of loss of activity of glucoamylase I (·–○–·) (1), the initial rate of destruction of tryptophanyl residues in glucoamylase I (––△––), and the initial rate of destruction of free L-tryptophan (—□—) (Fig. 2) during the photo-oxidation in the presence of Methylene Blue. Solutions, at each pH value, contained glucoamylase I (1 mg) and Methylene Blue (10 μg) in a total volume of 1.1 ml.

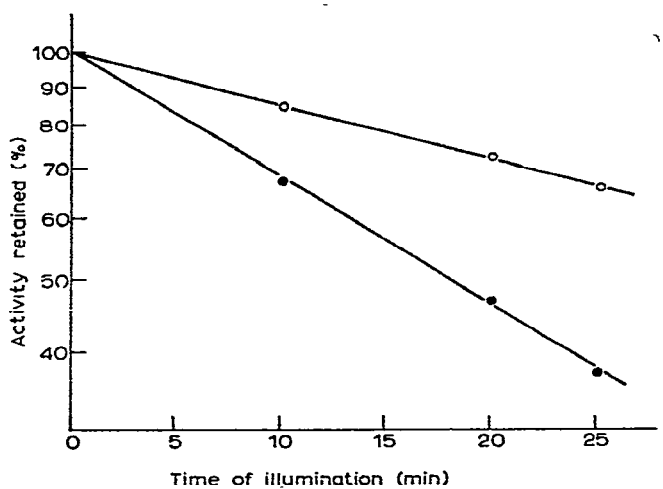

Fig. 4. The photo-oxidation of glucoamylase I in the presence of Rose Bengal at pH 4.5 (—○—) and 9.0 (—●—). Solutions, at each pH value, contained glucoamylase I (1 mg) and Rose Bengal (10 μg) in an initial volume of 1.1 ml.

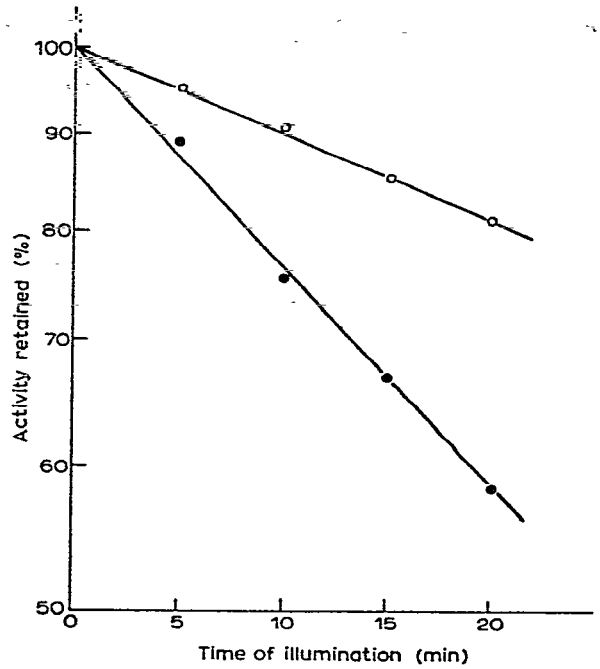

Fig. 5. The photo-oxidation of glucoamylase I with Rose Bengal in the presence (—○—) and absence (—●—) of 0.15M maltose, pH 4.5. Maltose (30 mg) was added to 0.6 ml of enzyme–dye solution (prepared as described for Fig. 4) immediately prior to photo-oxidation. The temperature of reaction was 10.8°.

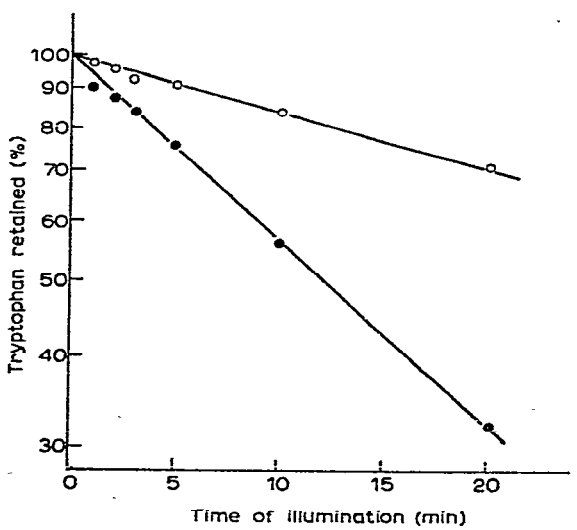

Fig. 6. The photo-oxidation of glucoamylase I, inactivated by reaction with glycine methyl ester in the presence of EDC, in the presence of Rose Bengal, in 0.15M maltose (—○—) and without maltose (—●—). Maltose (45 mg) was added to a solution of inactivated glucoamylase I (0.5 mg) and Rose Bengal (20 μg) in 0.07M acetate buffer (pH 4.5, 0.9 ml). The mixture was immediately illuminated in the usual way at 7.5°.

is present (~400%). Therefore, for substrate-inhibition studies, the former dye was employed as the sensitiser. As shown in Fig. 5, the presence of 0.15M maltose decreased the rate of loss of enzymic activity by 60.3%. In addition, the rate of loss of tryptophan, as determined by the method of Spies and Chambers[4], decreased by 59.3% (not shown).

We have recently reported the total inactivation of glucoamylase I by reaction of its carboxylic acid residues with glycine methyl ester in the presence of water-soluble ethyl(3-dimethylaminopropyl)carbodi-imide[2] (EDC). The results of photo-oxidation of glucoamylase I, so inactivated, in the presence of Rose Bengal in 0.15M maltose, are shown in Fig. 6. Maltose protected the tryptophanyl residues in the enzyme to an extent of ~69%, even though the enzyme was totally inactive.

DISCUSSION

The dye-sensitised photo-oxidation of tryptophan is complex and not fully understood. The products have not been fully quantitated but appear to belong to two main classes, namely melanines and kynurenine derivatives[8–12]. Simpler amino acids and a complex mixture of aromatic and polymeric materials may arise through secondary reactions[8,9]. It appears that photo-oxidation of tryptophan in proteins, in aqueous solution, results in kynurenine formation as the main pathway[8,9,11,12]. Following such a complex process by oxygen uptake would appear to be unsatisfactory. A more-satisfactory method is to follow the destruction of tryptophan by a chemical method[4]. Spectrophotometric methods, such as those of Bencze and Schmid[13] or Goodwin and Morton[14], cannot be employed to determine tryptophan, as *N'*-formylkynurenine, the major product of photo-oxidation of tryptophan, absorbs in the u.v. (λ_{max} 330 and 265 nm).

The results of such a study clearly indicate that the photo-oxidation of tryptophan in the presence of either Methylene Blue or Rose Bengal (cationic and anionic dyes, respectively) is pH-dependent, in support of the findings of other workers[15–17]. Westhead[3], however, reported that the photo-oxidation of tryptophan in the presence of Rose Bengal was not pH-dependent. However, oxygen uptake was employed and points were determined at only three pH values between 5.0 and 8.0. Weil[15], also using oxygen uptake, showed that the photo-oxidation of tryptophan in the presence of Methylene Blue was indeed pH-dependent. A curve similar to that in Fig. 2, up to pH 8.0, was obtained. A maximum between pH 8.0 and 8.5 was observed, however. Similar results were reported by Kochetov and Kobylyanska[16], who employed amino acid analysis to determine tryptophan. However, neither of these groups performed their experiments at a constant concentration of oxygen or determined true, initial, first-order rate constants. Weil[15] determined oxygen uptake after 30 min, whereas Kochetov and Kobylyanska[16] determined tryptophan after one hour of photo-oxidation. In the studies reported here, care was taken to ensure that true, initial, first-order rate constants were obtained under conditions of constant (saturated) oxygen concentration. This is most important, as determination of the

rate constants after a period of time has elapsed could produce erroneously low results due to such factors as irreversible dye oxidation, competition by the products for the activated dye and oxygen, and the consumption of oxygen. Indeed, a decrease in the rate constant was always observed in these studies as the time of reaction elapsed; this effect was most marked at higher pH values. The results presented here, based on true, initial rates, showed no evidence for a maximum in the pH-dependence of the initial rate constant for the reaction between pH 8.0 and 8.5, but a rapid increase was observed above pH 8.0. This was true whether Methylene Blue or Rose Bengal was used as the sensitiser.

The origin of the pH-dependence of the photo-oxidation of tryptophan is unknown, but it has been established that it is not due to ionisation of its carboxylic acid or amino groups, as glycyl-L-tryptophan and *N*-acetyl-L-tryptophanamide showed a pH-dependence similar to that of free L-tryptophan[15]. The pH-dependence of the destruction of free tyrosine and histidine during photo-oxidation in the presence of Methylene Blue is due primarily to the ionisation of the phenolic and imidazole side-chains, respectively, although an effect due to the α-amino group was noted in the latter amino acid[16]. For tryptophan, however, the pH-dependence is unlikely to be due to ionisation of the indole ring[18] (pK 16.82), as the curve is not consistent with a simple ionisation of this pK. The pH-dependence of this process may be due to the pH-dependence of the binding of the activated dyes to L-tryptophan, the pH-dependence of the formation or breakdown of the initially produced hydroperoxide[9], or to other processes.

We reported previously that the photo-oxidation of glucoamylase I from *A. niger* in the presence of Methylene Blue resulted in the destruction of tryptophanyl residues only, and a loss of enzymic activity[1].

In view of the above discussion, and in particular the results shown in Fig. 3, it is not now necessary to ascribe the pH-dependence of these processes to a conformational change in the protein, at least up to pH 7.0. Indeed, the correlation of the rates of loss of enzymic activity, destruction of tryptophanyl residues in the protein, and photo-oxidation of free tryptophan indicates that the loss of activity is related directly to the destruction of tryptophanyl residues, and that no conformational change takes place below pH 7.

In Fig. 3, above pH 8.0, the curves diverge, and although the pH-dependence of the rate of loss of activity corresponds well with that of the photo-oxidation of free tryptophan, the destruction of tryptophanyl residues in the protein is proportionately less rapid. Clearly, in this high pH range, other complicating processes must be occurring. The most probable explanation is that, at the higher pH values, a conformational change occurs which renders the tryptophanyl residues of the enzyme less exposed to the effects of the photo-oxidation. At the same time, those residues that are destroyed are such (possibly involved in the maintenance of the active three-dimensional structure) that their destruction gives rise to extensive loss of enzymic activity.

A correlation between loss of enzymic activity and photo-oxidation of free

tryptophan was also observed when Rose Bengal was used as photosensitiser (see Figs. 1 and 4, and Results). Therefore, it is likely that a similar phenomenon occurs in this case.

However, the results in Fig. 3 indicate that there are dangers in comparing losses of enzymic activity with destruction of *free* amino acids, especially at pH values far removed from neutrality.

In order to obtain information about the role of tryptophanyl residues in glucoamylase I, experiments were carried out in the presence of maltose, which is a low molecular weight substrate of the enzyme. 0.15M Maltose significantly protected the enzyme from photo-oxidation as Fig. 5 shows. Indeed, the protection of enzymic activity exactly parallels the protection of tryptophanyl residues from destruction (see Fig. 5 and Results), in that the rate of loss of enzymic activity decreased by 60.3% and the rate of destruction of tryptophanyl residues by 59.3%. Clearly, some of the tryptophanyl residues must lie in the active centre of the enzyme and it is likely that they are involved in the binding of the substrate.

This view is supported by the results obtained from photo-oxidation of glucoamylase that had been inactivated with glycine methyl ester and EDC[2]. The inactivation of the enzyme by this method is attributable to the reaction of carboxylic acid groups at the active centre. The observation that maltose protects the tryptophanyl residues in the *inactive* enzyme to a very significant extent from destruction by photo-oxidation indicates that the enzyme can still bind to the substrate even though it is catalytically inactive. Indeed, the protection afforded by maltose to the tryptophanyl residues in the inactivated enzyme (decreasing the rate of destruction by 69%) is of the same order as that afforded to the active enzyme (59.3%) under similar conditions.

The active centre of glucoamylase I from *A. niger* therefore appears to contain some tryptophan residues that participate in the binding of the substrate, while one or more of the carboxylic acid residues (there may be up to three[2]) are involved in the catalytic step in the action of the enzyme.

It has been reported[19] that the glucoamylase from *R. delemar* has an active site that binds many D-glucose residues (at least 15) and may be visualized as a cleft. The same may apply to glucoamylase I.

Tryptophanyl residues have been implicated in the binding of carbohydrate moieties to several proteins, including lysozyme[20–32], bacterial and porcine alpha-amylase[33–36], bacterial and viral neuramidase[37], *E. coli* galactose-binding protein[38], concanavalin A[39,40], pea phytohaemagglutinin[41], and a number of immunoglobulins[42,43]. The mode of binding of carbohydrates to the aromatic residues in proteins is not known. Besides simple hydrogen bonding, "hydrogen penetration", as invoked by Melcher to explain stabilisation of DNA and RNA double-helices[44], and double-stranded plane RNA[45], may be a factor. The hydrogen atoms on the carbohydrate rings may be bound to the π-electron system of the aromatic amino acid side-chains in the active centres of these proteins.

ACKNOWLEDGMENTS

The authors thank Professor S. A. Barker for his interest in this work, and the Science Research Council for a studentship (M.E.J.).

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