PHOTOOXIDATION OF GLUCOAMYLASE I FROM ASPERGILLUS NIGER

S. A. Barker, C. J. Gray and M. E. Jolley

Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham 15, England.

Received August 20, 1971

Summary Photooxidation of glucoamylase I in the presence of methylene blue results in the abolition of the enzymic activity. The process is pH- dependent, being more rapid at high pH. Loss of activity appears to be associated with destruction of tryptophan residues. The pH- dependence of the process is interpreted in terms of a conformational change undergone by the enzyme resulting in exposure of tryptophan residues which may be essential either directly for the activity or for the maintenance of the active three-dimensional structure.

INTRODUCTION

Photooxidation has often been used for the chemical modification of proteins in the study of structure-function relationships (1, 2, 3, 4, 5, 6). The amino-acid residues which are normally considered to be susceptible to modification by this technique are histidine, tryptophan, tyrosine, cysteine and methionine. By the use of this technique it has been possible in many cases to implicate a particular type of amino-acid residue, and even one individual residue, as being present at the active centre of an enzyme. For two enzymes in particular, aspartate amino-transferase (2) and enolase (3), the photooxidation process is pH- dependent and in each of these cases histidine residues were shown to be involved in

the catalytic site. The pH- dependences of these photooxidation were similar to that of the photooxidation of free histidine itself. We wish to report some studies on the photooxidation of a glucoamylase (α-1,4-glucan glucohydrolase, E.C. 3.2.1.3), a process which, although strongly pH-dependent, does not involve histidine residues.

MATERIALS AND METHODS

Crude glucoamylase from aspergillus niger (Agidex 3000) was purified by chromatography on DEAE cellulose, by a method similar to those of Lineback, Russell and Rasmussen(7) and Pazur and Ando(8). Two glucoamylase-active protein peaks were

TABLE

Amino-acid Composition of Glucoamylase I[a]

	A	B	C
Aspartic Acid	69	73	63
Threonine	72	75	81
Serine	77	81	90
Glutamic Acid	45	47	44
Proline	23	22	22
Glycine	47	47	45
Alanine	64	66	61
Valine	38	38	37
Cystine/2	7	7	5
Methionine	1.4	2	3
Isoleucine	22	21	22
Leucine	46	45	42
Tyrosine	26	27	23
Phenylalanine	22	21	22
Lysine	13	13	13
Histidine	4	4	4
Arginine	18	18	17
Tryptophan	19	-	22

a. Number of amino-acid residues based on a molecular weight of protein of 61,500 after Lineback(10).

A. The present preparation B. Lineback(10)
C. Pazur, Knull and Simpson(11)

obtained, that eluted second being identified as glucoamylase I(7,8) because of its elution behaviour on DEAE-cellulose, its higher electrophoretic mobility both in polyacrylamide gel and on cellulose acetate strips(9) and its amino-acid composition which is virtually identical to those quoted by Lineback(10) and by Pazur, Knull and Simpson(11) (see Table).

Photooxidation was carried out by illuminating solutions containing enzyme (1 mg/ml) and methylene blue (0.001%), previously saturated with oxygen, in a water-cooled apparatus with a 240 watt Phillips photoflood lamp at a distance of 15 cm. Samples were withdrawn at intervals and if necessary stored in light-proof tubes at 4° until required for activity determinations. Additional enzyme solutions photooxidised at pH 5.5, 7.8 or 9.0 for various times were taken for amino-acid analysis, tryptophan determination and activity measurements. Amino-acid analyses were obtained using a Technicon Autoanalyzer after acid hydrolysis under standard conditions. Tryptophan was determined by the method of Spies and Chambers(12).

Glucoamylase activities were determined from the release of glucose from starch (1% in 0.2M acetate buffer pH 4.5), liberated glucose being assayed by the glucose oxidase method of Dahlqvist(13).

RESULTS AND DISCUSSION

Initial rate constants for the inactivation of glucoamylase I on photooxidation at different pH values were obtained from first-order plots in the manner described by Westhead(3). Figure 1 shows that the rate of inactivation

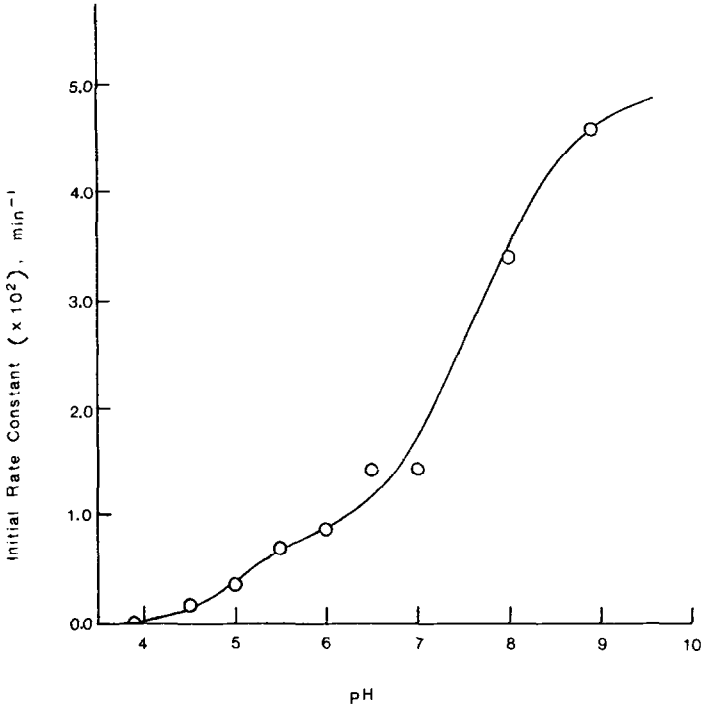

Fig. 1. Effect of pH on initial rate constant for inactivation of glucoamylase I on photooxidation.

---

varies markedly with pH. Westhead(3) reported that the inactivation of enolase by photooxidation in the presence of rose-bengal was pH- dependent, the variation being such that the process appeared to involve a single ionizing species with pK_a about 7. He also showed that the photooxidation caused destruction of histidine residues only. Since the photooxidation of free histidine exhibits a simple pH-dependence with a pK_a of about 7 it was concluded that a histidine residue essential to the enzyme's activity was destroyed and that the pH- dependence of the inactivation reflected the ionization of the imidazolium side-chain. A similar pH- dependence was observed for the photooxidation of aspartate amino-transferase in the presence of methylene

blue(2). In this case also, only histidine residues were destroyed.

In the case of glucoamylase I however, a different situation prevails. The shape of the pH- rate profile is not compatible with a simple single-proton ionization. In addition, amino-acid analysis of preparations photooxidized at pH 7.8 and 5.5 for various periods of time showed no significant destruction of histidine, or indeed of tyrosine or methionine. There was some evidence of destruction of half cysteine-residues although it did not follow a consistent pattern. It is unlikely that the thiol group of a cysteine residue is involved at the active site since we have demonstrated that the enzyme's activity is not affected by treatment with the thiol reagents p-chloromercuribenzoate (pH 9.0 or pH 4.5) or iodoacetate (pH's 4.5, 7.0 and 9.0). Indeed, the colorimetric method of Ellman(14) using 5,5'-dithio-bis(2-nitrobenzoic acid) gave no evidence of the presence of any thiol groups in the enzyme in either the absence or the presence of urea.

Photooxidation is clearly associated with loss of tryptophan. Figure 2 shows the loss of tryptophan with time on photooxidation at pH 5.5 and at pH 9.0. Also shown are the losses of activity during this experiment. We note that just as the loss of activity is slower at pH 5.5 than at pH 9.0, so also is the destruction of tryptophan. In addition photooxidation at pH 4.0 leads to the loss of neither tryptophan nor activity.

At pH 9.0, whereas the loss of activity follows a simple first-order reaction pattern, the experimental points for tryptophan destruction appear to lie on a curve corresponding

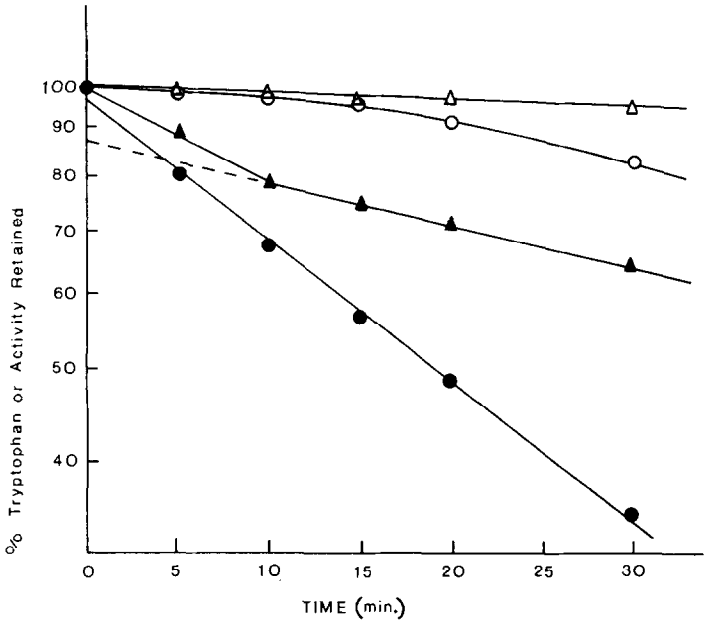

Fig. 2. Loss of activity and loss of tryptophan on photooxidation of glucoamylase I at pH 5.5 and at pH 9.0. Δ -tryptophan content, pH 5.5: O - activity remaining, pH 5.5; ▲-tryptophan content, pH 9.0; ● -activity remaining, pH 9.0.

to two straight lines in the log plot. Extrapolation of the second line (representing a slower reaction) back to zero time suggests that about 12% of the tryptophan residues in the protein (corresponding to approx. 2 residues) are destroyed relatively rapidly, the remainder being lost more slowly.

The results therefore, suggest that destruction of tryptophan residues by photooxidation results in loss of enzymic activity. This may mean that one or more tryptophan residues are present at the active site of the enzyme. This is the case for another glycoside hydrolase, lysozyme, in which chemical modifications have suggested the presence of essential tryptophan residues(15) and X-ray crystallographic studies have demonstrated the presence of tryptophan residues as substrate-binding sites in the enzyme(16). Alternatively

the tryptophan residues destroyed may be essential only for the maintenance of the correct three-dimensional structure.

The pH- dependence of the photooxidation process is unlikely to be due to simple ionization of exposed tryptophan residues, in contrast to the cases referred to above(2,3) in which the ionization of imidazolium groups of histidine residues was implicated. Sundberg(17) quotes pK_a values near -3.6 for the dissociation of the conjugate acid of indole. It would require a very unusual environment to raise this to the value of 7-8 which would be compatible with Fig. 1. It seems more likely that the increased susceptibility to photooxidation of the tryptophan residues is due to their increased exposure at higher pH values. In other words, as the pH is increased, the protein molecule unfolds allowing previously inaccessible residues to be attacked. The conformational change at high pH is presumably not drastic and is reversible since in all controls readjustment back to the pH for activity measurements gave preparations with full activity.

ACKNOWLEDGEMENTS

We are grateful to Professor M. Stacey, F.R.S. for his interest in this work, to the S.R.C. for a studentship (to M.E.J.) and to Professor Lineback for allowing us to quote his results in the table.

REFERENCES

1. Weil, L., Buchert, A. R. and Maher, J., Arch. Biochem. Biophys., 40, 245 (1952); Weil, L. and Seibles, T. S., Arch. Biochem. Biophys. 54, 368 (1955).

2. Martinez-Carrion, M., Turano, C., Riva, F. and Fasella, P., J. Biol. Chem., 242, 1426 (1967).

3. Westhead, E. W., Biochemistry, 4, 2139 (1965).

4. Ingram, J. M. and Hochster, R. M., Can. J. Biochem, 45, 929, 1505 (1967).

5. Hoffee, P., Lai, C. Y., Pugh, E. L. and Horecker, B. L., Proc. Natn. Acad. Sci., U.S.A. 57, 107 (1967).

6. Koshland, D. E., Jr., Strumeyer, D. H. and Ray, W. J., Jr., Brookhaven Symposia in Biology, No. 15, p. 101 (Brookhaven National Laboratory, Upton, New York, 1962).

7. Lineback, D. R., Russell, I. J. and Rasmussen, C., Arch. Biochem. Biophys., 134, 539 (1969).

8. Pazur, J. H. and Ando, T., J. Biol. Chem., 234, 1966 (1959).

9. Webb, E. C., Nature (London), 203, 821 (1964).

10. Lineback, D. R., Personal Communication.

11. Pazur, J. H., Knull, H. R. and Simpson, D. L., Biochem. Biophys. Res. Commun., 40, 110 (1970).

12. Spies, J. R. and Chambers, D. C., Anal. Chem., 21, 1249 (1949).

13. Dahlqvist, A., Biochem. J., 80, 547 (1961).

14. Ellman, G. L., Arch. Biochem. Biophys., 82, 70 (1959).

15. Hartdegen, F. J. and Rupley, J. A., Biochim. Biophys. Acta, 92, 625 (1964); Hayashi, K., Imoto, T. and Funatsu, M., J. Biochem (Tokyo), 54, 381 (1963); Hachimori, Y., Horonishi, H., Kurihara, K. and Shibata, K., Biochim. Biophys. Acta, 93, 346 (1964).

16. Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C. and Sarma, V. R., Proc. Roy. Soc., 167B, 365 (1967); Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C. and Sarma, V. R., Proc. Roy Soc., 167B, 378 (1967); Johnson, L. N., Phillips, D. C. and Rupley, J. A., Brookhaven Symposia in Biology, No. 21, p. 120 (Brookhaven National Laboratory, Upton, New York, 1968).

17. Sundberg, R. J., "The Chemistry of Indoles", p. 5, Academic Press, New York, 1970.