BBA 63485

Chemical modification of bacterial α-amylase by monoiodoacetate

The role of several sorts of amino acid residues of α-amylases in their catalytic activity has been studied by chemical modification techniques as well as kinetic methods[1–4]. Possible participation of the histidyl residue as an essential group of α-amylase has been suggested from the study of pH effect on the rate of enzyme reaction[5]. An alkylating reagent has been successfully used by CRESTFIELD *et al.*[6,7] for the chemical modification of essential histidyl residue of ribonuclease. In this paper, chemical modification of bacterial α-amylase with monoiodoacetate was investigated in order to probe the sort of amino acid residue which is related to the catalytically active state of the enzyme. It was found that no amino acid residue was modified at pH 5.5 and 40° in acetate buffer. However, methionyl residues were modified by monoiodoacetate in phosphate and even in acetate buffer after removal of calcium. The result is discussed in relation to the role of methionyl residues and calcium in the enzyme molecule.

Bacterial liquefying α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) from *Bacillus subtilis* was purchased from Daiwa Kasei Co., Osaka and used without further purification. The enzyme preparation contained 5.0% calcium and $6.9 \cdot 10^{-2}$% zinc, and any protein other than bacterial α-amylase was not contained in this enzyme preparation. Iodine-free monoiodoacetate was obtained by recrystallization from chloroform. Hydrolysis of the enzyme for analysis of amino acid composition was carried out in 6 M HCl at 110° for 22 h. Amino acid analysis was performed with a Hitachi KLA-3 Amino Acid Analyzer according to the method of SPACKMAN *et al.*[8]. The enzyme activity toward soluble starch (0.1%) was determined at pH 5.5, 40° by reducing value method[9]. The chemical modification reactions were commenced by adding monoiodoacetate to the enzyme solution (0.28 mM) at 40°, and aliquots were removed for the determination of the remaining activity after definite time intervals. Calcium of the enzyme preparation was removed by column chromatography (Sephadex G-50, 0.2 M acetate buffer, pH 5.5).

When the chemical modification with monoiodoacetate was carried out in

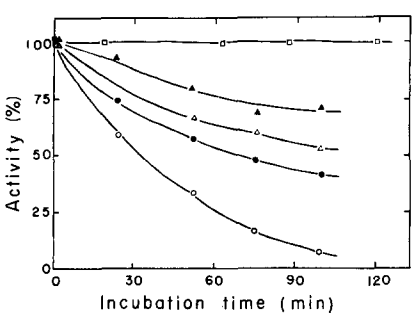

Fig. 1. Effect of monoiodoacetate on enzyme activity. The reactions of chemical modification were carried out at 40° in 0.2 M phosphate buffer, pH 5.5. Activity (%) represents the relative rate of the enzyme reaction of the modification reaction mixture at the indicated time of incubation. Bacterial α-amylase: 0.28 mM. Monoiodoacetate: ○—○, 1.0 M; ●—●, 0.5 M; △—△, 0.25 M; ▲—▲, 0.13 M; □—□, 0 M (control).

TABLE I

AMINO ACID COMPOSITION OF BACTERIAL α-AMYLASE TREATED WITH MONOIODOACETATE IN PHOSPHATE AND ACETATE BUFFER AT pH 5.5

The number of amino acid residues was calculated on the basis of a molecular weight of 47 500 for bacterial α-amylase and of 29 for alanyl residues of the enzyme. ppt, the precipitated enzyme; sup, the enzyme in the supernatant. Activity (%) represents the relative rate of the enzyme reaction of the modification reaction mixtures. Tryptophyl residues were determined by the colorimetric method[14].

Reaction system	*In phosphate buffer*					*In acetate* buffer*
Monoiodoacetate (M)	*0*	*0.5*		*0.75*	*1.0*	*1.0*
Sample having activity of (%)	*100 (intact)*	*50*		*25*	*0*	*0*
Preparation	—	*ppt*	*sup*	*ppt*	*ppt*	—
Aspartic acid	53	53	52	50	53	53
Threonine	22	23	22	22	24	22
Serine	24	25	25	24	25	24
Glutamic acid	41	42	41	42	44	42
Proline	16	15	16	17	15	16
Glycine	38	38	36	37	38	38
Alanine	29	29	29	29	29	29
Valine	23	24	24	22	24	24
Methionine	5.5	3.9	5.0	2.4	1.7	5.0
Isoleucine	16	16	16	16	16	16
Leucine	21	22	22	19	21	22
Tyrosine	23	24	24	24	24	24
Phenylalanine	16	17	17	17	17	17
Lysine	25	27	27	25	25	25
Histidine	12	11	12	11	11	12
Arginine	18	18	18	18	17	17
Tryptophan	16	—	—	—	—	16

* Results obtained without removal of calcium and in 0.2 M acetate buffer, pH 5.5, at 40° for 150 min.

0.2 M phosphate buffer, pH 5.5, a marked inactivation of the enzyme was observed, as is shown in Fig. 1. Rate and extent of the inactivation were closely related to the concentration of monoiodoacetate. Therefore, various enzyme preparations having the desired degree of inactivation were obtained by varying the concentration of monoiodoacetate after a definite period (100 min) of incubation. In the course of the inactivation, precipitates appeared, the amounts of which were proportional to the concentration of monoiodoacetate. Amino acid composition of the enzyme protein in the precipitates and the supernatant are shown in Table I. The precipitates were isolated from the reaction mixture by centrifugation, washed with distilled water, and lyophilized. Only methionyl residues of the precipitated enzyme are found to react with monoiodoacetate, other amino acid residues not reacting, as is shown in Table I. The amount of methionyl residues and their derivatives, which are produced from methionyl residue during acid hydrolysis of the enzyme samples[10,11], are presented in Table II. A total amount of methionine and its derivatives is almost equal to that

TABLE II

DETERMINATION OF METHIONYL RESIDUE AND ITS DERIVATIVES

Table represents methionyl residue and its derivatives of the precipitated enzyme in the modification reaction mixture, which have 100 (intact), 50, 25, and 0% of the remaining activity, respectively (see Table I).

Sample having activity of / *Residue* (%)	*100 (intact)*	*50 ppt*	*25 ppt*	*0 ppt*
Homoserine	0	0.51	1.10	1.17
Homoserine lactone	0	0.62	0.78	1.03
Carboxymethylhomocysteine	0	0.62	0.78	1.35
Methionine	5.5	3.90	2.40	1.70
Total	5.5	5.65	5.06	5.25

of the intact enzyme. This clearly indicates that the modification reaction of methionyl residue with monoiodoacetate proceeds stoichiometrically. Decrease of methionyl residues coincides with loss of activity. Three methionyl residues of the completely inactivated enzyme were modified, whereas the intact α-amylase contained five methionyl residues. Methionyl residue of the enzyme in the supernatant was found to remain unmodified. Specific activities (4.0–5.0 glucose(γ)/enzyme(γ) per min) of the enzymes in the supernatant were nearly equal to that (4.5 unit) of the intact enzyme. Therefore, the enzyme in the supernatant was intact and the precipitated enzyme was inactive. It was not confirmed that the enzyme in the precipitates had well-defined components, because the precipitates were found to be insoluble in an aqueous solution. These results suggest that the chemical modification of three methionyl residues of bacterial α-amylase with monoiodoacetate is intimately related with the denaturation which leads to the precipitation of the enzyme. Accordingly, these three methionyl residues may be essential in the maintenance of the active state of the enzyme

It has been reported that calcium bears a role in the maintenance of active state of bacterial α-amylase molecule[12,13]. These experimental results obtained as above in phosphate buffer may be interpreted as follows: The removal of calcium is caused by phosphate ion, and methionyl residues are exposed. The exposed methionyl residues are modified with monoiodoacetate, which leads to precipitation (denaturation) of the enzyme. This interpretation was supported by the following experiments.

Without using phosphate buffer, after removal (decrease) of calcium through column chromatography (calcium content decreased to 2.0%; control, 5.0%), the chemical modification using monoiodoacetate was carried out in 0.2 M acetate buffer, pH 5.5. The result of chemical modification of this preparation by monoiodoacetate was the same as that obtained by using phosphate buffer as described. In addition, another experimental result which supports the conclusion is: When the enzyme was treated with monoiodoacetate in 0.2 M acetate buffer, pH 5.5, which does not remove calcium, inactivation of the enzyme was also observed. But no precipitate appeared in this case. Amino acid composition of the inactivated enzyme was determined after elimination of monoiodoacetate by column chromatography with Sephadex G-50. Amino acid composition of the fully inactivated enzyme (remaining activity, 0%), which was treated by monoiodoacetate for 150 min, was almost equal to that of intact bacterial α-amylase, as is shown in the last column in Table I. None of the amino acid

residues is modified by monoiodoacetate. These results distinctly support the above supposition.

By the treatment with monoiodoacetate, methionyl residues of bacterial α-amylase were chemically modified and inactivation and denaturation of the enzyme were observed in phosphate buffer and even in acetate buffer with the removal of calcium. In acetate buffer without removal of calcium, the chemical modification of methionyl residue and denaturation (precipitation) of the enzyme were not observed.

These results show that chemical modification of methionyl residues of bacterial α-amylase with monoiodoacetate brings about denaturation (precipitation) of the enzyme, and the modification reaction is inhibited by calcium. It may be possible that these methionyl residues are located in the binding sites of the calcium to the enzyme. These three methionyl residues may play an essential role in the maintenance of the catalytically active state of the enzyme molecule in cooperation with calcium.

Department of Chemistry, Faculty of Science, MASATAKE OHNISHI
Kyoto University, Kyoto (Japan) HIROYUKI HATANO

1 K. SUGAE, *J. Biochem. Tokyo*, 48 (1960) 790.
2 T. YAMAMOTO, *Bull. Agr. Chem. Soc. Japan*, 19 (1955) 121.
3 Y. OKADA, K. ONOUE, S. NAKASHIMA AND Y. YAMAMURA, *J. Biochem. Tokyo*, 54 (1963) 447.
4 J. WAKIM, M. ROBINSON AND J. A. THOMA, *Carbohydrate Res.*, 10 (1969) 487.
5 S. ONO, K. HIROMI AND Y. YOSHIKAWA, *Bull. Chem. Soc. Japan*, 31 (1948) 957.
6 A. M. CRESTFIELD, W. H. STEIN, AND S. MOORE, *J. Biol. Chem.*, 238 (1963) 2413.
7 A. M. CRESTFIELD, W. H. STEIN, AND S. MOORE, *J. Biol. Chem.*, 238 (1963) 2421.
8 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
9 K. HIROMI, Y. TAKAHASHI AND S. ONO, *Bull. Chem. Soc. Japan*, 36 (1963) 563.
10 H. G. GUNDLACH, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 234 (1959) 1754.
11 H. G. GUNDLACH, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 234 (1959) 1761.
12 T. YAMAMOTO, *Bull. Agr. Chem. Soc. Japan*, 20 (1956) 188.
13 T. YAMAMOTO AND J. FUKUMOTO, *Bull. Agr. Chem. Soc. Japan*, 23 (1959) 68.
14 J. R. SPIES AND D. C. CHAMBERS, *Anal. Chem.*, 21 (1949) 1249.

Received July 28th, 1970