BBA 66492

THE REACTIVATION OF PHENYLMETHANESULFONYL-SUBTILISIN

NOBUO UEMITSU, MINEKAZU SUGIYAMA AND HIROYUKI MATSUMIYA

Life Sciences Laboratory, Nomura Research Institute of Technology and Economics, 1600 Kajiwara, Kamakura, Kanagawa (Japan)

(Received August 9th, 1971)

SUMMARY

1. Phenylmethanesulfonyl fluoride-inactivated subtilisin is reactivated by formohydroxamic acid and hydroxylamine. Formohydroxamic acid causes reactivation to an extent of 100%.

2. From the observation that the pH–activity profile of reactivation of the inactivated subtilisin by formohydroxamic acid was S-shaped, it is concluded that the reactivation is analogous to the deacylation step of the enzyme–substrate reaction.

3. Diisopropylfluorophosphate-inactivated subtilisin is not reactivated by formohydroxamic acid.

INTRODUCTION

We take interest in structure–function relationships of proteolytic enzymes and, in connection with this, the problem whether the structure as a whole is necessary for enzyme activity. One of the methods we adopted to study this problem is enzymatic fragmentation of subtilisin BPN′ by trypsin, by which active fragments of lower molecular weights maintaining the enzyme activity might be yielded if there is no necessity for the complete native structure. Prior to tryptic fragmentation of subtilisin, it has to be inactivated so that it does not hydrolyze trypsin and subtilisin itself. The inactivated subtilisin is reactivated after tryptic fragmentation. Studies of reversible inhibitors of subtilisin, therefore, are essential to our purpose. We report some results obtained in the course of attempts to reactivate the inactivated subtilisin.

Diisopropylfluorophosphate (DFP)-inactivated serine proteases, diisopropylphosphoryl (DIP)-trypsin and DIP-chymotrypsin[1,2] are known to be reactivated by nucleophilic reagents[3–5]. Subtilisin is inactivated by DFP[6,7], but so far reactivation has not been observed. Phenylmethanesulfonyl fluoride (PMSF) reacts with subtilisin to form enzymatically inactive phenylmethanesulfonyl (PMS)-subtilisin[8–10].

Abbreviations: DFP, diisopropylfluorophosphate; DIP-, diisopropylphosphoryl-; PMSF, phenylmethanesulfonyl fluoride; PMS-, phenylmethanesulfonyl-; DEP-, diethylphosphoryl-.

MATERIALS AND METHODS

Three times crystallized bacterial alkaline poteinase, subtilisin BPN', was purchased from Nagase and Co., Ltd.; PMSF was from Eastman Organic Chemicals; DFP and *N*-acetyl-L-tyrosine ethyl ester were from Sigma Chemical Co.; hydroxylamine·HCl was from Wako Pure Chemical Industries, Ltd.; Osaka, formohydroxamic acid was prepared by the method of HICKENBOTTOM[11].

The reaction of PMSF ($2.53 \cdot 10^{-4}$ M) with subtilisin ($4.4 \cdot 10^{-5}$ M) was carried out in 0.05 M Tris–maleate buffer, pH 8.0, containing 0.01 M Ca^{2+}. The reaction mixture was dialyzed for 48 h at 4° against the same buffer. 0.5 ml of the resultant PMS-subtilisin ($3.75 \cdot 10^{-5}$M) was added to 0.5 ml of 1 M formohydroxamic acid, adjusted to the proper pH. Aliquots were drawn for assay of activity at appropriate time intervals. *N*-Acetyl-L-tyrosine ethyl ester was used as substrate in all subtilisin assays. The concentration of the components in the reaction mixture was 0.01 M substrate, 0.1 M KCl and 8% (v/v) dimethylsulfoxide. The reaction rates were determined with the aid of a Radiometer Model TTT1c pH stat with a Model SBR2 recorder and a thermostated reaction vessel. Titration was performed at pH 8.0 and 37° with 0.02 M NaOH.

RESULTS AND DISCUSSION

Native subtilisin was incubated with nucleophilic reagents in a control experiment; the activity of subtilisin was not affected by the presence of those reagents for 6 h at pH 5.0–10.0. PMS-subtilisin slowly regained activity when incubated in the absence of reagents. The extent of spontaneous reactivation became higher as the solution was made more alkaline. The extent of reactivation was, however, less than 1.0%, even at pH 8.5, within 5 h.

Fig. 1 shows the time course of reactivation of PMS-subtilisin by 0.5 M formohydroxamic acid in 0.05 M Tris buffer containing 0.01 M Ca^{2+}, pH 8.5, at 25°. PMS-subtilisin was reactivated linearly up to 3.5 h and was completely reactivated after 6 h. 0.5 M hydroxylamine, on the other hand, reactivated PMS-subtilisin to the extent of 10% even after 15 h incubation at pH 9.0. Those results are similar to those for diethylphosphoryl-α-chymotrypsin (DEP-α-chymotrypsin) which is reactivated

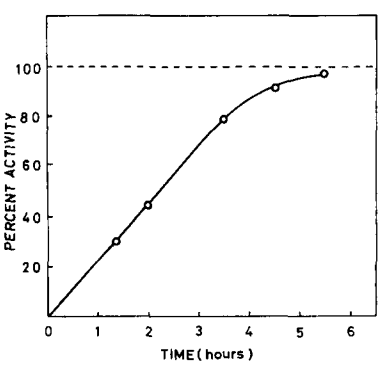
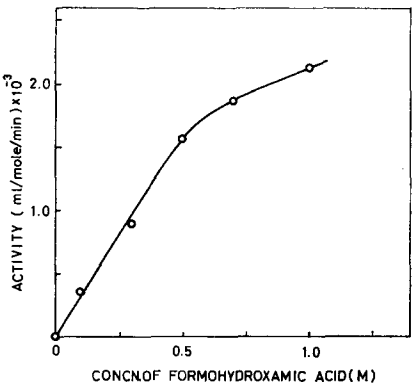


Fig. 1. Time course of reactivation of PMS-subtilisin (1.04 mg/ml) by 0.5 M formohydroxamic acid. Buffer, 0.05 M Tris buffer, pH 8.5, containing 0.01 M Ca^{2+}; temperature, 25°.

completely by formohydroxamic acid and to an extent of 13.5% by 1.04 M hydroxylamine at pH 7.0 in 15 h (ref. 3).

The extent of reactivation of PMS-subtilisin (1.04 mg/ml) in the presence of increasing concentrations of formohydroxamic acid (0.05 M Tris buffer; 0.01 M Ca^{2+}; pH 8.5; 25°; incubation time, 2 h) is shown in Fig. 2. The reactivation increased linearly up to a concentration of 0.5 M formohydroxamic acid.



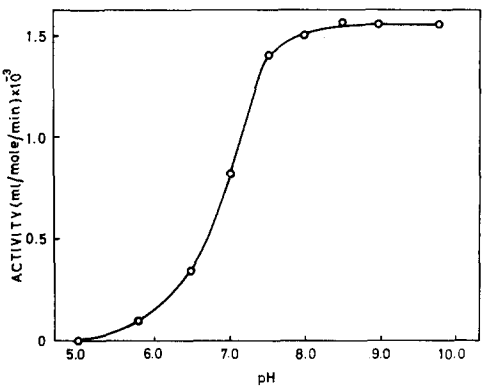


Fig. 2. Effect of formohydroxamic acid concentration upon reactivation of PMS-subtilisin. Time, 2 h; conditions as described in the legend to Fig. 1.

Fig. 3. Effect of pH upon reactivation of PMS-subtilisin by 0.5 M formohydroxamic acid. Time, 2 h; temperature, 25°; buffer, same as in Fig. 1.

The effect of pH upon the reactivation of PMS-subtilisin by 0.5 M formohydroxamic acid is shown in Fig. 3. The curve is S-shaped and is different from those found for the reactivation of DEP-α-chymotrypsin[5] and DEP-trypsin[4], which are bell shaped with optima at pH 8.15 and 7.00, respectively. This S-shaped curve is in accord with the data reported by STEWARD AND OUELLET[12] for the deacetylation of monoacetyltrypsin and also agrees with findings of BENDER *et al.*[13] for the deacylation of cinnamoyl-α-chymotrypsin. Hence, in the case of PMS-subtilisin, its reactivation is similar to deacylation.

An attempt to reactivate DIP-subtilisin failed. Incubation of DIP-subtilisin with 0.5 M formohydroxamic acid at pH 8.0 for 25 h did not recover the enzyme activity. It is an interesting problem why DIP-subtilisin is not reactivated by formohydroxamic acid, whereas DEP-α-chymotrypsin and DEP-trypsin are.

ACKNOWLEDGEMENTS

Our thanks are due to Mr. F. Saeki, the president of Nomura Research Institute, for his interest and encouragement of our work, to Professor E. Ichishima of Tokyo University of Agriculture and Technology and Mr. H. Murakami for their valuable advice for the present work.

REFERENCES

1 E. F. JANSEN, D. NUTTING, R. JANG AND A. K. BALLS, *J. Biol. Chem.*, 179 (1949) 189.
2 R. A. OOSTERBAAN, P. KUNST AND J. A. COHEN, *Biochim. Biophys. Acta*, 16 (1955) 299.

3 L. W. CUNNINGHAM, *J. Biol. Chem.*, 207 (1954) 443.
4 W. COHEN, M. LACHE AND B. F. ERLANGER, *Biochemistry*, 1 (1962) 686.
5 W. COHEN AND B. F. ERLANGER, *J. Am. Chem. Soc.*, 82 (1960) 3928.
6 M. OTTESEN AND C. G. SCHELLMANN, *C. R. Trav. Lab. Carlsberg, Sér. Chim.*, 30 (1957) 157.
7 H. MATSUBARA AND S. NISHIMURA, *J. Biochem. Tokyo*, 45 (1958) 503.
8 L. POLGAR AND M. L. BENDER, *J. Am. Chem. Soc.*, 88 (1966) 3153.
9 E. N. KENNETH AND D. E. KOSHLAND, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1606.
10 L. POLGAR AND M. L. BENDER, *Biochemistry*, 6 (1967) 610.
11 W. J. HICKENBOTTOM, *Reactions of Organic Compounds*, Longmans, Green and Co., New York, 1959, p. 301.
12 J. A. STEWART AND L. OUELLET, *Can. J. Chem.*, 37 (1959) 751.
13 M. L. BENDER, G. R. SCHONBAUM, G. A. HAMILTON AND B. ZERNER, *J. Am. Chem. Soc.*, 83 (1961) 1255.