REACTIVATION OF α-CHYMOTRYPSIN INHIBITED BY ORGANIC PHOSPHATES

by

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Since the first demonstration by JANSEN and coworkers[1] of the inhibition of α-chymotrypsin by di*iso*propyl fluorophosphate (DFP) and its analogs, no method of reactivation of the enzyme has been found[2]. The success of WILSON in reactivating acetylcholine esterase, inhibited by tetraethylpyrophosphate (TEPP) and DFP, with hydroxylamine and related compounds[3] has led us to attempt similar studies on α-chymotrypsin.

Inhibited enzyme was prepared by allowing a 0.1% solution of twice recrystallized α-chymotrypsin to react with diethyl-*p*-nitrophenylphosphate (DNP)* in a tris(hydroxymethyl)aminomethane-HCl buffer at pH 8. The course of the reaction was followed spectrophotometrically by determining the absorption at 400 mμ of the nitrophenolate liberated by the inhibition reaction[4]. The inhibited protein was twice crystallized according to the method of KUNITZ for α-chymotrypsin[5] and was found to be 99.6% inhibited.

Reactivation was attempted in 0.1 *M* $CaCl_2$ at varying concentrations of hydroxylamine over a wide pH range. All activity measurements were made with acetyl-L-tyrosine ethyl ester as substrate[6], in the presence of 0.1 *M* $CaCl_2$. Since aliquots of the reactivation mixture were used directly to determine chymotrypsin activity, the effect of hydroxylamine on esterase determinations was measured. The highest amine concentration prevailing in activity measurements was 0.2 *M* and was found to increase the apparent esterase activity not more than 5 to 10%. This effect, presumably due to the synthesis of acetyl-L-tyrosinhydroxamide, as revealed by a positive ferric chloride test[7], was not large enough to affect the interpretation of the extent of reactivation of the inhibited enzyme.

In 1 *M* hydroxylamine between pH 6 and 8, the inhibited enzyme was slowly reactivated, approaching a maximum of 25–35% reactivation after 72 hours. In 2 *M* hydroxylamine, reactivation was accelerated but did not exceed about 40%. Under the same experimental conditions, α-chymotrypsin retained its full activity, whereas in the absence of hydroxylamine no reactivation of the inhibited enzyme occurred. Measurements of the effect of pH on the rate of reactivation by 1 *M* hydroxylamine, within the pH range of 5.4 to 9.4, showed a maximum at pH 6.7 to 7.0. The data could be quantitatively accounted for on the assumption that hydroxylamine had interacted with some group of the inhibited enzyme which has a pK of about 7.8. Crystalline, DFP-inhibited α-chymotrypsin could likewise be partially reactivated at pH 7.0 though after 50 hours in 1 *M* hydroxylamine not more than 5% reactivation could be measured.

Further studies on the mechanism of reactivation are in progress.

REFERENCES

1 E. F. JANSEN, M.-D. NUTTING, R. JANG AND A. K. BALLS, *J. Biol. Chem.*, 179 (1949) 189; E. F. JANSEN, M.-D. F. NUTTING AND A. K. BALLS, *ibid.*, 179 (1949) 201.
2 A. K. BALLS AND E. F. JANSEN, *Advances in Enzymol.*, 13 (1952) 321.
3 I. B. WILSON, *J. Biol. Chem.*, 190 (1951) 111; *ibid.*, 199 (1952) 113.
4 B. S. HARTLEY AND B. A. KILBY, *Biochem. J.*, 50 (1952) 672.
5 J. H. NORTHROP, M. KUNITZ AND R. M. HERRIOTT, *Crystalline Enzymes*, 2nd. Ed., Columbia University Press, New York (1948).
6 S. KAUFMAN, H. NEURATH AND G. W. SCHWERT, *J. Biol. Chem.*, 177 (1949) 793.
7 R. B. JOHNSTON, M. J. MYCEK AND J. S. FRUTON, *J. Biol. Chem.*, 185 (1950) 629.

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