STUDIES ON THE ACTIVE SITE OF α-CHYMOTRYPSIN

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The sequence of the amino acids in the phosphopeptides derived from α-chymotrypsin, inhibited by diisopropyl phosphofluoridate (DFP), has been reported (Oosterbaan et al., 1958). Subsequent investigations (Viswanatha, 1964) suggested the presence of a basic component with an "activated guanido" group in the active site of α-chymotrypsin. These studies have now been extended to establish the identity of the basic amino acid as well as to elucidate its mode of attachment within the phosphopeptide. The present report concerns the nature of this basic component present in the phosphopeptide derived from α-chymotrypsin.

Materials and Methods: 3x recrystallised chymotrypsin was purchased from Worthington Biochemicals Ltd., Freehold, New Jersey. 5x recrystallised ribonuclease was obtained from General Biochemicals, Chargin Falls, Ohio. DFP was a product of the Aldrich Chemical Company, Milwaukee.

The phosphopeptide was prepared according to the procedure described by Viswanatha (1964).

Reduction of the phosphopeptide with $LiBH_4$ was performed in tetrahydrofuran according to the procedure of Chibnall and Rees,(1958).

The amino acid content of the phosphopeptide was determined by hydrolysis for 18 hrs. with constant boiling HCl followed by analysis according to Spackman et al.,(1958) using a Beckman model 120-B amino acid

analyzer. The guanido amino acid in the reduced phosphopeptide and in α-chymotrypsin was determined quantitatively using the Sakaguchi reaction (Greenstein & Winitz, 1961). The arginine content of α-chymotrypsin was also determined chromatographically in the HCl hydrolysate of the protein (Spackman et al., 1958).

Results: Figure 1 shows that the HCl hydrolysate of the 3x chromatographed phosphopeptide revealed no basic amino acid although equimolar amounts of aspartic acid, serine and glycine were detected. However, reduction of the phosphopeptide with $LiBH_4$, results in the formation of an amino acid emerging after ammonia on column chromatographic analysis (Spackman et al., 1958). This basic component was isolated and found to give a positive Sakaguchi reaction. Similar results were obtained when the phosphopeptide was reduced with HI and red phosphorus. This basic amino acid, formed on reduction of the phosphopeptide, emerged at the same effluent volume as synthetic γ-hydroxy arginine upon ion-exchange chromatography and underwent facile lactonization in acidic solution like the latter amino acid (Santarossa, 1967). Quantitative estimation of the basic component by the Sakaguchi reaction revealed the presence of 0.9 moles of the guanido compound per mole of other amino acids present in the peptide.

In the light of the above results the arginine content of chymotrypsin and chymotrypsinogen were determined by the quantitative Sakaguchi reaction, using arginine and 5x recrystallised ribonuclease as standards. The intensity of the colour formed in the case of arginine, ribonuclease and chymotrypsinogen diminished slowly, while the intensity obtained with chymotrypsin increased steadily reaching a maximum value 10 minutes after the addition of hypobromite. Hence all absorbance measurements were made 20 minutes after the addition of the hypobromite reagent. When thus analysed chymotrypsin was found to contain 3.94 moles of arginine per mole of protein. In contrast, amino acid analy-

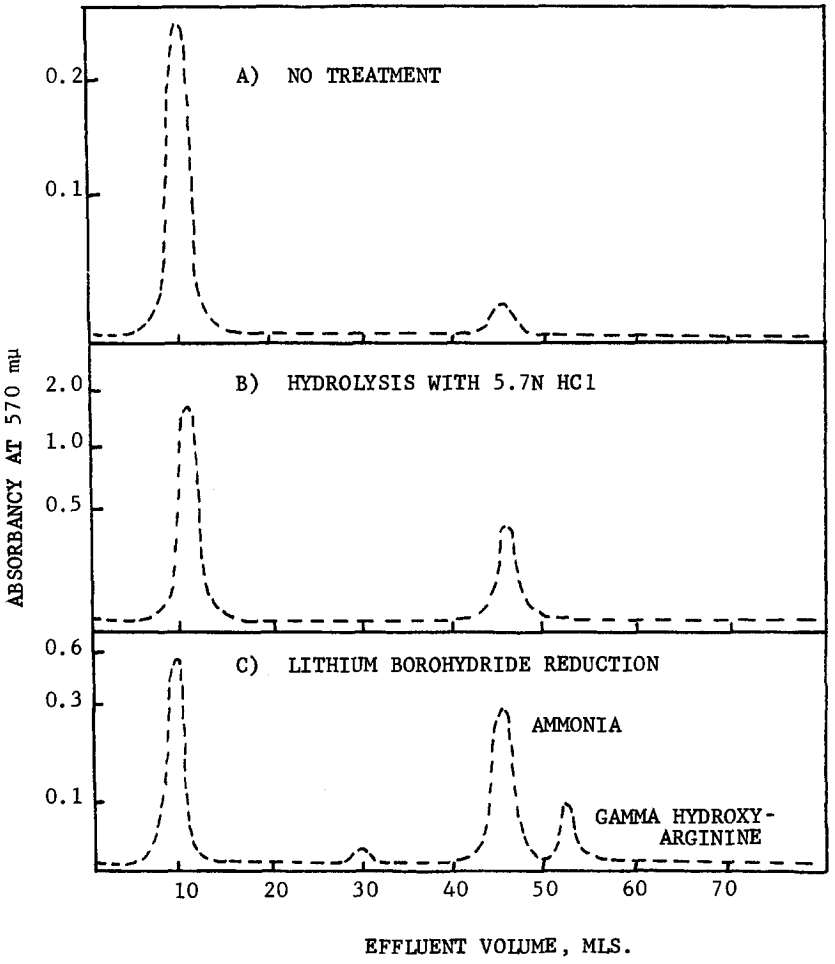

Figure 1: Chromatographic behaviour of the phosphopeptide under various conditions. 7.6 cm. column, 0.38N sodium citrate buffer pH 5.28, temperature 55°C, flow rate 60 ml per hour.

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sis of the acid hydrolysate of chymotrypsin showed only 3.01 moles of arginine per mole of protein in agreement with published reports (Viswanatha and Lawson, 1961; Walsh and Neurath, 1964); these results are shown in Table I.

TABLE I

ARGININE CONTENT OF PROTEINS

PROTEIN[a]	Moles of Arginine per mole Protein	
	Observed	Expected[b]
Ribonuclease	4.00[c]	4.0
Chymotrypsinogen	3.92[c]	4.0
Chymotrypsin	3.94[c]	3.0
	3.01[d]	

a. protein concentrations were estimated spectrophometrically using the $E_{cm}^{1\%}$ value cited by Edelhoch (1967);
b. taken from literature (see text);
c. based on quantitative Sakaguchi reactions;
d. based on amino acid analysis.

Discussion: Reduction of the phosphopeptide by $LiBH_4$ consistently results in the formation of a basic amino acid which resembles γ-hydroxy arginine in its column chromatographic behaviour. It should be noted however, that the acidic conditions used to decompose excess of the reductant, favor the formation of the lactone of the amino acid which adheres to the column. Hence the quantitation of the basic amino acid cannot be achieved by ion exchange chromatography. Direct estimation of the guanido group by the Sakaguchi reaction provides the best approach for the quantitation of the basic amino acid released upon reduction.* Such analyses show the presence of nearly 1.0 mole of guanido compound per mole of other amino acids present in the peptide. Based on its column chromatographic behaviour, this residue can be tentatively identified as γ-hydroxy arginine. It appears therefore, that the amino acid composition of the phosphopeptide is (Asp, Ser, Gly, x, $PO_4^{=}$) with

* Some preparations of the phosphopeptide have been found to yield positive Sakaguchi reactions. No concrete explanations can be offered for this phenomenon at the moment; but the possibility of N→O phosphoryl migration involving γ-OH group of the arginine being responsible for the observation is under investigation.

x being the precursor of γ-hydroxyarginine. The reasons for the failure on the part of earlier investigators to detect this amino acid have been discussed (Viswanatha, 1964).

The Sakaguchi procedure used for the quantitative estimation of arginine shows the presence of one additional mole of guanido compound per mole of chymotrypsin, not detected in the normal amino acid analyses (Table I). The agreement noted in the arginine content of ribonuclease as measured by the Sakaguchi reaction, with that of amino acid analysis (Hirs et al, 1956), attests to the reliability of the former technique. The additional "arginine" residue in chymotrypsin found, using the Sakaguchi procedure, may explain the observation of Marini and Wunsch (1963) who have reported the presence of a titratable, strongly cationic, presumably guanido group, not apparent in the known amino acid composition of the enzyme. This additional guanido group may well represent the postulated guanidoxy linkage, between the serine and the arginine residues (Viswanatha, 1964), which, by virtue of its similarity to desaminocanavanine, would show positive Sakaguchi reaction (Kitagawa and Tsukamoto, 1937).

Since the activation of chymotrypsinogen to chymotrypsin involves the removal of an arginine residue (Dreyer and Neurath, 1955) the zymogen should contain an additional mole of this amino acid. However, identical arginine contents are noted in the zymogen and the enzyme (Table I). The relationship of this observation to the structural changes associated with the zymogen activation is under investigation.

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REFERENCES

Chibnall, A.C. and M.W. Rees, Biochem. J. 68, 105 (1958).
Dreyer, W.J. and H. Neurath, J. Am. Chem. Soc. 77, 814 (1955).
Edelhoch, H. Biochemistry, 6, 1948 (1967)
Greenstein, J.P. and M. Winitz, in "Chemistry of the Amino Acids," p.1847 J. Wiley & Sons.
Hirs, C.H.W., S. Moore and W.H. Stein. J. Biol. Chem. 219, 623 (1956).
Kitagawa, M. and J. Tsukamoto, J. Biochem. (Japan) 26, 373 (1937).
Marini, M.A. and C. Wunsch, Biochemistry 2, 1454 (1963).
Oosterbaan, R.A., P. Kunst, J. Van Rotterdam and J.A. Cohen, Biochim. Biophys. Acta. 27, 550 (1958).
Santarossa, B.A., M.Sc. Thesis, University of Waterloo (1967).
Spackman, D.H., W.H. Stein and S. Moore, Anal. Chem. 30, 1190 (1958)
Viswanatha, T. and W.B. Lawson, Arch. Biochem. Biophys. 93, 128 (1961).
Walsh, K. and H. Neurath, Proc. Natl. Acad. Sci. (U.S.) 52, 884 (1964).
Viswanatha, T. Proc. Natl. Acad. Sci., 51, 1117 (1964).