IDENTIFICATION OF THE METHIONINE INVOLVED IN THE ACTIVE CENTER OF CHYMOTRYPSIN

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Ray et al. (1, 2) have recently applied careful kinetic methods in following the methylene blue catalyzed photo-oxidation of chymotrypsin (3) and have shown that concomitant with activity loss one of the two methionine residues and one of the two histidine residues are rapidly oxidized.

It has now been possible to locate the methionine that is rapidly and preferentially photo-oxidized as that three residues distant from the serine which is labelled by diisopropylfluorophosphate. The second methionine which is 15 residues removed from this serine is only slowly photo-oxidized.

Hartley (4) has obtained a tryptic peptide (Fig. 1) from S-sulfo-chymotrypsinogen which contains the "active center" serine as well as both methionine residues of chymotrypsin. We began the present work by preparing this peptide from S-sulfo-DIP^{32}-α-chymotrypsin but it was observed that spontaneous oxidation of the methionine residues during preparation led to multiple forms of this peptide. Stabilization of methionine was achieved by alkylation to the sulfonium salt with bromo - or iodoacetic acid in the presence of urea (5). Since only free methionine and not its oxidized form is alkylated, it was possible to label the non-oxidized methionine residue with Br$C^{14}H_2COOH$ and thus determine the position of oxidized

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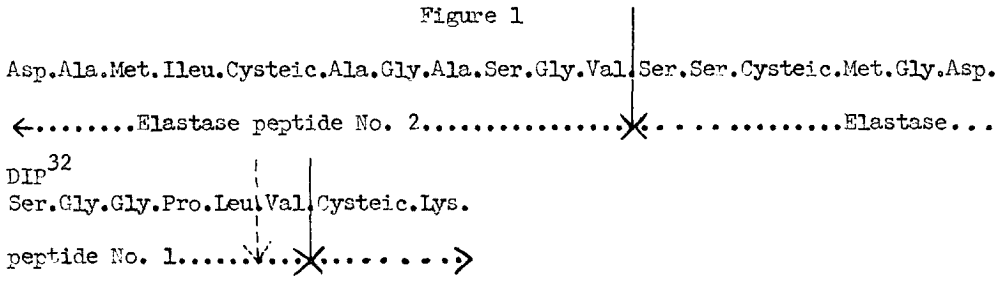
methionine by comparison of the peptide prepared from photo-oxidized chymotrypsin with the peptide from native chymotrypsin.

Figure 1



Experimental

400 mg of α-chymotrypsin (Worthington 3x cryst. Lot 6011A+B) was dissolved in 80 ml of 0.05M phosphate buffer at pH 7.2 with 0.01% methylene blue (1, 2). 40 ml of the solution in a 1 litre Erlenmeyer flask was exposed for 10 minutes to the light from four 375-watt lamps (Sylvania movie light EBR). Light entered through the bottom of the flask which was immersed in a glass water-bath kept at 5°C. Aliquots of 0.025 ml were withdrawn every minute and assayed for chymotryptic activity against acetyl tyrosine ethyl ester at pH 8.0, 25°C.

The solutions, after removal of methylene blue with charcoal, were dialyzed at 4°C for 24 hours against several changes of 0.001M hydrochloric acid and lyophilized. The residues were dissolved in 5 ml of 8 molar urea adjusted to pH 2.7 with hydrochloric acid in the presence of 0.01M $ICH_2C^{14}OOH$ (0.38 millicuries per millimole), and incubated at 40°C for 24 hours. The solutions were then dialyzed extensively for 40 hours at 4°C against several changes of 0.001M hydrochloric acid and lyophilized. The dried material was dissolved in performic acid (1 volume 30% hydrogen peroxide to 9 volumes 99% formic acid) which had been pre-incubated for 2 hours at room temperature. Oxidation was for 3 hours at 4°C and the reaction was stopped by dilution with water and lyophilization (twice). The proteins were then digested with trypsin (0.16 micromoles) for 22 hours at 25°C after solution in 25 ml of 0.12M

ammonium acetate buffer at pH 8.5 (0.05 molar in calcium chloride). After acidification to pH 3.0 (formic acid) and centrifugation the supernatant solution was concentrated and "finger-printed" (high voltage electrophoresis at pH 6.5, 3Kv for 30 minutes, followed by paper chromatography in n-butanol-acetic acid-water), 3:1:1. From 80 to 84 per cent of the C^{14} was in a single peptide (localized by radioautography) with amino acid composition Asp_2, Ser_{3-4}, $Prol_1$, Gly_5, Ala_3, $Cysteic_3$, Val_2, $Meth_2$, $Ileu_1$, Leu_1, Lys_1. This labelled peptide, corresponding to Hartley's "active center" peptide (Fig. 1), was purified by preparative high voltage electrophoresis at pH 6.5. The specific activity of the purified peptides from control and photo-oxidized chymotrypsin was determined by amino acid analysis and liquid scintillation counting. Table 1 indicates results from two separate experiments. There is excellent agreement between our results and the predicted values based on the kinetic data of Ray et al. (1, 2).

The distribution of the C^{14} activity between the two methionine residues was determined by digestion of the radioactive peptides with a purified elastase preparation (kindly supplied by R. Donovan and C. S. Hanes of this Department). About 0.4 micromoles of peptide from control and photo-oxidized chymotrypsin were subjected to about 0.003 micromoles of elastase at pH 8.5 (0.05M ammonium acetate) in a total volume of 2 ml at 25°C for 12 hours. After evaporation, the residue was taken up in water and subjected to high voltage electrophoresis at pH 3.6 (3Kv, 30 minutes). Radioautography revealed two major C^{14} peptides from both control and photo-oxidized samples. These peptides were counted as above, eluted from the paper, hydrolyzed and analysed for amino acid content.

The results in Table 2 indicate that the methionine residue nearest the "active center" serine (elastase peptide No. 1) is photo-oxidized far more rapidly than the other methionine residue. The methionine sulfoxide content can be determined either from the specific activity data or from the methionine sulfone content (6) but the latter method gave lower results.

TABLE 1

	Expt. 1	Expt. 2
Per cent inactivation	82%	85%
Rate constant of inactivation by photo-oxidation	0.17 min^{-1}	0.21 min^{-1}
Alkylating agent	$BrC^{14}H_2COOH$	$ICH_2C^{14}OOH$
Specific activity of purified "active center" peptide cpm/μm		
Control	54,400	380,000
Photo-oxidized	34,200	219,000
Increase in sulfoxide* on photo-oxidation	$\frac{544-342}{544} \times 100\%$ = 37.1%	$\frac{380-219}{380} \times 100\%$ = 42.3%
Methionine sulfone content of purified "active center" peptide		
Control	0.09 residues	0.08 residues
Photo-oxidized	0.61 residues	0.53 residues
% increase in sulfone on photo-oxidation (Neumann method)	$\frac{0.61-0.09}{2.0} \times 100\%$ = 26.0%	$\frac{0.53-0.08}{2.0} \times 100\%$ = 22.5%

* Ray et al (1, 2) would predict about 40% oxidation of the methionine at 80% inactivation.

Discussion

Since the "fast" methionine is only 3 residues removed from the serine involved in the "active center", it must be very close to this serine in space, particularly if this region of the polypeptide chain is α-helical in the native enzyme. Two consequences of the close proximity of the methionine and serine residues become apparent. First, since the serine is involved in the enzymatic process and appears to bind acyl groups during catalysis, it must be accessible to substrate molecules and exposed on the surface of the protein. It is likely, therefore, that the "fast" methionine is also located at the surface of the protein and readily accessible to the

TABLE 2

	Control Sample			Photo-oxidized Sample		
Elastase peptide	No. 1	No. 2	(1+2)	No. 1	No. 2	(1+2)
cpm/μM x 10^{-3}	194	187	381	35.4	183	218
% non-oxidized methionine	51.0	49.0	100	9.3	48.0	57.3
% methionine sulfoxide (increase on photo-oxidation)	--	--	--	41.7	1.0	42.7
Amino Acid Residues						
Methionine sulfone	0.07	0.02	0.09	0.61	0.10	0.71
Aspartic	1.2	1.0	2.2	1.2	1.0	2.2
Serine	2.5	1.1	3.6	2.3	1.0	3.3
Proline	1.1	0.1	1.2	1.0	0.1	1.1
Glycine	2.8	1.9	4.7	2.6	1.8	4.4
Alanine*	0.7	2.4	3.1	0.9	2.4	3.3
1/2-Cystine	0.9	1.0	1.9	1.2	1.0	2.2
Valine	0.4	1.0	1.4	0.5	1.0	1.5
Isoleucine	0.1	0.7	0.8	0.1	0.7	0.8
Leucine	0.9	0.1	1.0	0.9	0.1	1.0
Electrical charge	-1	-2	--	-1	-2	--
% increase in sulfone on photo-oxidation (Neumann method)	--	--	--	27.0	4.0	31.0

* There is a possible discrepancy between our results and the sequence reported by Hartley for his "active center" peptide (Fig. 1). Hydrolytic cleavages at Val-Ser and Val-Cysteic should produce a C^{14} methionine peptide with 3 alanine residues and a C^{14} peptide without alanine. Our results indicate that one of the alanines may be incorrectly placed in Hartley's sequence.

oxidizing radicals produced in the solvent during photo-oxidation. The second methionine which is 15 residues away might be buried much further in a non-aqueous region of the molecule. Secondly, since the "fast" methionine is so close to the serine, any alteration of its chemical properties would be expected to modify the behaviour of the serine and thus exert a profound

effect on enzyme activity. It seems clear from the work of Richards and Vithayathil with RNAase (7) that the oxidation of the methionine in the S-peptide leads to a profound alteration in the binding of the S-peptide to the S-protein. This has been attributed to the destruction of a hydrophobic interaction due to the change from the hydrophobic methionine residue to the hydrophilic methionine sulfone residue. In the case of chymotrypsin the formation of methionine sulfoxide (also hydrophilic) could cause inactivation of the enzyme either if the methionine were part of a postulated specificity site at which the hydrophobic R groups of specific substrates are bound or if the methionine were essential for the maintenance of secondary and/or tertiary structure of the molecule and hence the orientation of the active site.

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