Tetrahedron Vol. 41, No. 5, pp. 901 to 905, 1985
Printed in Great Britain.

0040-4020/85 $3.00 + .00

SEMISYNTHESIS II - FORMATION OF A COVALENTLY LINKED 1-12/106-129 -FRAGMENT OF HEN EGG-WHITE LYSOZYME.

I.J. Galpin* and D.A. Hoyland

The Robert Robinson Laboratories, The University of Liverpool, P.O.Box 147, Liverpool, L69 3BX, England

(*Received in UK* 2 *November* 1984)

Abstract: Methoxycarbonylsulphenyl chloride has been shown to cleave reduced Hen egg-white lysozyme into two fragments, rather than the 1-12, 13-105 and 106-129 fragments which would have been anticipated. The covalently linked 1-12/106-129 and 13-105 fragments are produced by initial cleavage at methionines 12 and 105 resulting in the generation of two homoserine lactone residues in place of the methionines. The homoserine lactone at position 12 then couples to the alpha amino group of asparagine-106 to give a covalently linked 1-12/106-129 fragment which has been characterised by a variety of techniques.

The general features of protein semisynthesis were outlined in the previous paper,[1] and in the present work we were interested in modifying the freely available hen egg white lysozyme in such a way as to provide analogues of the enzyme.

The initial plan was to reduce hen egg white lysozyme and to protect the eight cysteine sulphydryl functions prior to digestion with cyanogen bromide. As hen egg white lysozyme contains only two methionine residues, cleavage with cyanogen bromide should give three fragments, that is the 1-12, 13-105 and 106-129 fragments.[2] Indeed, earlier work[2] demonstrated that cleavage at methionines 12 and 105 could be achieved and after disulphide interchange with mercaptoethanol that three fragments could be obtained. As a result of the cyanogen bromide cleavage, the methionines at positions 12 and 105 would be converted to homoserine lactone residues and it was proposed that these activated fragments could be used in semisynthesis allowing the assembly of an enzyme analogue by incorporation of synthetic peptide fragments. This general approach has been used on several occasions for other enzymes,[3] but at present no semisynthetic analogue of lysozyme has been prepared. Other workers have, however, used tryptic fragmentation of the enzyme to provide fragments which were reassembled giving large sub-fragments.[4] Our approach using cyanogen bromide would have the advantage that only three fragments would be generated, whereas using trypsin considerably more fragments would be obtained, as cleavage occurs at each of the seventeen basic residues.

Hen egg white lysozyme was treated with dithiothreitol to reduce the disulphide bridges.[5] The resulting reduced hen egg white lysozyme was shown to be homogeneous by electrophoresis and had a satisfactory amino acid analysis.

The reduced protein was then treated with methoxycarbonyl sulphenyl chloride,[6] this reagent has been shown to give a stable non-symmetrical disulphide which can be utilised in further synthesis by reaction with other thiols. The use of this reagent was demonstrated elegantly in the work of the Ciba Geigy group in their synthesis of human insulin.[7] We envisaged the use of this reagent, as it would provide protection for the sulphydryl groups, but at a later stage could be used as an activating group for the reformation of disulphide bonds.

Treatment of the Scm protected, reduced, hen egg white lysozyme with cyanogen bromide for fifteen hours in 70% formic acid followed by dilution and chromatography on Sephadex G50 gave two components with Ve/Vt 0.37 and 0.56. Although these results obtained by U.V. detection at 280 nm indicate that only two products were produced, it is possible that a third component was present as the 1-12 fragment would not shown an absorption at this wavelength. The amino acid analysis (Figure 1) of the two peaks indicated that the first fraction (Ve/Vt 0.37) was clearly

FRAGMENT	1-12/106-129		13-105	
	A	B	A	B
Trp	3	Nd	3	Nd
Lys	2	2.30	4	4.09
His	0	0	1	0.85
Arg	5	4.76	6	6.72
Asp	3	2.74	18	15.36
Thr	1	0.87	6	5.38
Ser	0	0	10	7.79
Pro	0	0	2	2.23
Glu(Hse)	2	2.27	3	3.10
Gly	3	3.19	9	8.89
Ala	6	5.95	6	7.02
½Cys	3	Nd	5	Nd
Val	3	3.00	3	3.58
Met	1	0	1	0.11
Ile	1	0.91	5	4.68
Leu	2	1.85	6	5.75
Tyr	0	0	3	3.21
Phe	1	1.06	2	1.94

Figure 1 Amino-acid analysis of reduced Hen egg-white lysozyme after treatment with methoxycarbonylsulphenyl chloride and fractionation on Sephadex G50. 1-12/106-129 has Ve/Vt 0.56 and 13-105 has Ve/Vt 0.37. A - Theoretical composition of fragment; B - values found on acid hydrolysis; Nd - indicates residue not determined.

the central 13-105 portion of the enzyme and that the second peak (Ve/Vt 0.56) contained all the amino acids present in the 1-12 and 106-129 fragments. Fractions eluting after this point were pooled and subjected to amino acid analysis, but no peptidic material was evident. The experiment was repeated omitting the cyanogen bromide treatment and the same two fragments were observed, thus confirming that the methoxycarbonylsulphenyl chloride was able to cleave the peptide chain in a manner similar to cyanogen bromide.[1]

Our initial reaction was that the 1-12 and 106-129 fragments had co-eluted. However, electrophoresis using a urea based buffer at pH 7.0 showed both peaks obtained from gel filtration on Sephadex G50 to be homogeneous. The material with Ve/Vt 0.56 was then applied to Sephadex G25 and again a homogeneous peak (Ve/Vt 0.69) was obtained. On the basis of these results it may be concluded that the fragments 1-12 and 106-129 must have become covalently linked during the digestion procedure.

It is possible that the link between the fragments could be due to the presence 6-127 disulphide bridge, either resulting from the digestion procedure, or remaining from the original reduction of hen egg white lysozyme with dithiothreitol, although it is claimed that under the conditions employed total reduction of the enzyme occurs.[5]

A second alternative was that amide bond formation had occurred between the activated homoserine lactone formed during chain cleavage and either the amino terminus of the 106-129 fragment or the side chain amino function of lysine-116. These possibilities were then examined in detail to establish the nature of the linkage between the 1-12 and 106-129 fragments.

The 1-12/106-129 hybrid was subjected to simultaneous reduction and sulphitylation, this procedure should reduce any disulphide bonds present including the disulphide of the Scm protection giving a sulphitylated product. Thus, if the linkage between the two fragments 1-12 and 106-129 were through a disulphide bond then this bond would be broken and the 1-12 and 106-129 fragments would be liberated. If, however, the linkage between the two fragments was not through a disulphide, then a sulphitylated 1-12/106-129 material would be produced. The product

of sulphitylation was chromatographed on Sephadex G25 eluting with 50% acetic acid, giving a symmetrical peak with Ve/Vt of 0.69, having the same amino acid analysis to the original material.

As a further test Scm chloride bearing a ^{13}C label in the methoxyl group was used for digestion. The 1-12/106-129 hybrid material resulting in this case showed the presence of a labelled methoxyl group in the hybrid product, although in this and the new 13-105 product, which was also isolated, a rather broad signal due to the ^{13}C methoxyl group was observed. Thus, the magnetic environment on individual cysteine residues is not sufficiently different to allow resolution of individual ^{13}C signals for the methoxyl groups in different environments. On the basis of these tests we concluded that a disulphide bond was not implicated in the linkage of the 1-12 and 106-129 fragments.

The 1-12/106-129 hybrid was then subjected to alkaline hydrolysis in order to eliminate any possibility of an ester linkage being present between the two fragments. Gel filtration on Sephadex G25 of the hydrolysate showed an identical peak to that obtained before hydrolysis at Ve/Vt 0.69. Furthermore, this peak had the same amino acid analysis and electrophoretic mobility to the original 1-12/106-129 hybrid.

An N-terminal analysis using the Dansyl technique[8] was then carried out on both the 1-12/106-129 and 13-105 fragments. N-terminal lysine was confirmed for both fragments, but the 1-12/106-129 hybrid showed N-terminal lysine and no other Dansyl labelled amino acid, thus the asparagine at position 106 could not be confirmed.

As stated in the previous paper,[1] we found that digestion of octasulphityl hen egg white lysozyme with cyanogen bromide and digestion of reduced hen egg white lysozyme with Nps chloride both gave three fragments. Thus, the reaction in the case of Scm chloride digestion is wholly exceptional.

Two possibilities exist for the formation of a 1-12/106-129 hybrid. The first is that the amino group at position 106 is in close proximity to the homoserine lactone generated at position 12. Inspection of a model of the enzyme indicates that in the intact protein residues 12 and 106 are within a few angstroms. It is thus just possible that this proximity is maintained in the reduced enzyme, although it is appreciated that total disorganisation of the tertiary structure would normally occur on reduction of the disulphide bonds. A second explanation could lie in the temporary formation of a 6-127 disulphide linkage, although the involvement of cysteine 115 is also possible. If such a disulphide were produced, either from the linear sequence of reduced hen egg white lysozyme, or from the separate 1-12 and 105-129 fragments, then the amino terminal 106 position could be brought into close proximity to the homoserine lactone at position 12, thus generating what is an intramolecular situation. The deliberate use of existing disulphide bonds has been used in the case of BPTI[9] to generate a homoserine analogue employing the disulphide to hold the two fragments in position, and as will be shown in the following paper[10] a similar approach may be used for the formation of lysozyme analogues. In this case the disulphide would need to be transient as no disulphide is present in the final product.

As is generally accepted, methoxycarbonylsulphenyl chloride is used to protect thiol functions and subsequently to generate non-symmetrical disulphide links. However, we found that if native hen egg white lysozyme was treated with this compound then small quantities of 1-12 and 106-129 fragments were liberated. Due to the positioning of the two methionine residues and four disulphide bonds, it is necessary to cleave both the peptide chain and the disulphide bridges in order to liberate these fragments. In the previous paper,[1] we have shown that methoxycarbonylsulphenyl chloride is able to cleave peptide chains containing methionine, thus in order to liberate the 1-12 and 106-129 fragments cleavage of the disulphide bonds must also occur with this reagent.

In order to confirm this finding, a sample of bovine insulin was treated with excess Scm chloride, as insulin does not contain any methionine no complications due to the presence of this residue would occur. In this experiment gel filtration on Sephadex G50 allowed the isolation of pure B-chain, although the A-chain was contaminated with undigested insulin.

This finding confirms that disulphides may be cleaved in some circumstances by excess Scm chloride.

The formation of a bridged 1-12/106-129 fragment and a 13-105 fragment from reduced hen egg white lysozyme on treatment with Scm chloride has, therefore,been demonstrated, and the linkage between the 1-12 and the 106-129 fragments appears to be through an amide bond connecting homoserine-12 with asparagine-106. As cleavage of methionyl peptide bonds by sulphenyl chlorides had been demonstrated in the previous paper[1] the linkage of the 1-12 and 106-129 fragments must have taken place by attack of the alpha amino function of asparagine-106 on the homoserine lactone at position 12 in the 1-12 fragment.

EXPERIMENTAL

Materials and general techniques.

Hen egg-white lysozyme and reagents were purchased from the sources indicated in the preceeding paper,[1] except that 5-dimethylaminonaphthalene-1-sulphonyl chloride and bovine insulin were obtained from BDH Chemicals and dried cells of *micrococcus lysodeikticus* were obtained from the Sigma chemical company. Thin layer chromatograms of dansyl derivatives were run on polyamide plates, being visualised by UV fluorescence.

Treatment of reduced hen egg-white lysozyme with methoxycarbonylsulphenyl chloride and followed by cyanogen bromide digestion.

Reduced hen egg-white lysozyme[5] (0.1g, 6.95 μM) was dissolved in anhydrous formic acid (50 ml) and treated with methoxycarbonylsulphenyl chloride[6] (8.8 mg, 69.5 μM) for 3 hours. The solvent was evaporated *in vacuo* and the residue dissolved in 70% formic acid (2 ml) prior to desalting by gel filtration on Sephadex G25 eluting with 70% formic acid. The material contained in the major peak was isolated after lyophilisation and was redissolved in 70% aqueous formic acid. Cyanogen bromide (37 mg, 349 μM) was added and the solution stirred for 15 hours. Water (100 ml) was then added and the solution lyophilised. The product was subjected to gel filtration on Sephadex G50 eluting with 70% formic acid, two major peaks were observed at Ve/Vt 0.37 and 0.56, amino-acid analysis of the material isolated from these peaks is given in Figure 1.

Digestion of reduced hen egg-white lysozyme with methoxycarbonylsulphenyl chloride.

Reduced hen egg-white lysozyme[5] (0.1g, 6.95 μM) was dissolved in anhydrous formic acid (50 ml) and methoxycarbonylsulphenyl chloride[6] (8.8 mg, 69.5 μM) added. After stirring for three hours the solvent was evaporated *in vacuo* and the residue dissolved in a mixture of formic acid (2 ml) and water (0.9 ml). Gel filtration on Sephadex G50 in 70% formic acid was then carried out and the major peaks eluted with Ve/Vt 0.37 and 0.56. Electrophoresis on cellulose acetate, 0.1M phosphate, pH 7/8M urea (1:7), p.d. 1.5 KV, 10 mA developed with Ponceau S gave (Ve/Vt 0.37 material) -0.2 and (Ve/Vt - 0.56 material) -0.84, mobilities relative to native hen egg-white lysozyme -1.0.

Sulphitylation of peptides/proteins containing disulphide bonds.[11]

The peptide/protein (5.2 μM) was dissolved in freshly deionised 8M urea solution (15 ml) and 1M trihydroxymethylmethylamine (Tris)/HCl buffer, pH 7.5 (1.5 ml) added. Anhydrous Na_2SO_3 (620 mg, 5mM) was dissolved in this solution and oxygen passed through the solution for 20 minutes. The pH was adjusted to 7.5 with 1M HCl and a single crystal of cysteine hydrochloride (ca. 5 mg) added. After stirring for 18 hours at 37° the solution was exhaustively dialysed against distilled water using Visking tubing. The residual solution was then lyophilised.

Preparation of (^{13}C-methoxy)-carbonylsulphenyl chloride.[12]

Chlorocarbonylsulphenyl chloride (2.03g, 16 mM) and (^{13}C)-methanol (0.5g, 16 mM) were dissolved in Et_2O (10 ml) and gently warmed to 30°; triethylamine (2.18 ml, 16 mM) was then added and the solution stirred for 3 hours. The solution was filtered and the filtrate warmed to 40° whilst dry nitrogen was bubbled through to evaporate the Et_2O. The crude product was distilled *in vacuo* with the distillate being collected at 30° (11 mmHg), yielding the required product (1.24g, 70%), ^{1}H nmr ($CDCl_3$) {d, J(^{13}C-H), 151.2 Hz; δ 4.0}, ^{13}C nmr ^{1}H decoupled ($CDCl_3$) CH_3(s); δ 55.75, carbonyl not observed.

Alkaline hydrolysis of the putative 1-12/106-129 fragment.

A sample of the so called 1-12/106-129 fragment (30 mg) was dissolved in 2M NaOH at pH 12 and the solution stirred at room temperature for 15 hours. The pH of this solution was then adjusted to 7 with acetic acid and the resulting solution evaporated *in vacuo* to give a residue which was applied to Sephadex G25 and eluted with 50% aqueous acetic acid. A single symmetrical peak was observed at Ve/Vt 0.69 and after pooling of the fractions and lyophilisation the solid residue was subjected to 6M acid hydrolysis and amino-acid analysis. The amino-acid analysis of this material was unchanged from the analysis which was performed prior to the treatment with base.

N-Terminal analysis using Dansyl derivatisation.[8]

The peptide (ca. 5 μM) was dissolved in 0.5M $NaHCO_3$/8M urea (20 ml) and 5-dimethylamino-naphthalene-1-sulphonyl chloride (Dansyl chloride) (2.7 mg, 10 μM) in the minimum volume of acetone added. The solution was stirred at 37° for 2 hours then placed in Visking tubing and dialysed exhaustively against distilled water. The resulting solution was frozen and lyophilised giving a solid residue which was subjected to standrad acid hydrolysis. The acid was evaporated *in vacuo* to give a residue which was dissolved in EtOAc. A sample of this solution was then applied to a two-sided polyamide tlc plate and the two-way chromatogram developed solvent 1 : aqueous formic acid (1.5% v/v); solvent 2 : toluene/glacial AcOH 9/1 v/v. Comparison with authentic bis Dnp-lysine and Dnp aspartic acid run on the reverse side of the plate was then made.

Digestion of native Hen egg-white Lysozyme with methoxycarbonylsulphenyl chloride

Hen egg-white lysozyme (0.1g, 6.95 μM) was dissolved in anhydrous formic acid (20 ml) and methoxycarbonylsulphenyl chloride (350 mg, 2.77 mM) added. The solution was stirred for 3 hours, distilled water (8.5 ml) added and stirring continued for 15 hours. The solvent was evaporated *in vacuo* and the residue dissolved in 98% formic acid (1.75 ml) and distilled water (0.75 ml) added. The resulting hazy solution was filtered, diluted with distilled water, and lyophilised. The residue was then subjected to gel filtration on Sephadex G50 eluting with 25% aqueous acetic acid.

Digestion of bovine insulin with methoxycarbonylsulphenyl chloride.

Bovine insulin (40 mg, 6.9 μM) was dissolved in anhydrous formic acid (10 ml) and methoxycarbonylsulphenyl chloride (0.13 ml, 1.38 mM) added. After 15 hours the solvent was evaporated *in vacuo* and the residue dissolved in 70% formic acid (2ml) prior to gel filtration on Sephadex G50 eluting with 70% formic acid. Fractions making up the peaks with Ve/Vt 0.33, 0.45 and 0.53 were pooled and lyophilised. The material isolated in each case was characterised by amino-acid analysis. (Ve/Vt 0.45, B-chain), Lys(1) 1.00, His(2) 2.00, Arg(1) 1.00, Asp(1) 1.02, Thr(1) 0.97, Ser(1) 0.80, Pro(1) 0.90, Glu(3) 3.26, Gly(3) 3.14, Ala(2) 2.10, ½Cys(2) Nd, Val(3) 3.12, Leu(4) 4.23, Tyr(2) 1.94, Phe(3) 2.74, (Ve/Vt 0.33 whole insulin), (Ve/Vt 0.53 contaminated A-chain).

Acknowledgement - We wish to thank the S.E.R.C. for a studentship to D.A.H.

REFERENCES

1. I.J.Galpin and D.A.Hoyland, *Tetrahedron* submitted for publication, Semisynthesis I.
2. B.Bonavida, A.Miller and E.E.Sercarz, *Biochemistry*, 8, 968 (1969).
3. R.E.Offord and C.DiBello, Semisynthetic peptides and proteins, Academic Press, London (1978).
4. A.R.Rees and R.E.Offord, *Biochem.J.*, 159, 487 (1976).
5. T.A.Bewley and C.H.Li, *Int.J.Peptide Protein Res.*, 1, 117 (1969).
6. R.G.Hiskey, N.Muthukumaraswamy and R.R.Vunnam, *J.Org.Chem.*, 40, 950 (1975).
7. P.Sieber, B.Kamber, A.Hartmann, A.Johl, B.Riniker and W.Rittel, *Helv.Chim.Acta*, 60, 27 (1977).
8. W.R.Gray, *Methods Enzymology*, 25, 121 (1972).
9. D.F.Dyckes, H.Kini and R.C.Sheppard, *Int.J.Peptide Protein Res.*, 9, 340 (1977).
10. I.J.Galpin and D.A.Hoyland, *Tetrahedron*, submitted for publication, Semisynthesis III.
11. A.R.Rees and R.E.Offord, *Biochem.J.*, 159, 467 (1976).
12. K.Nokihara and H.Berndt, *J.Org.Chem.*, 43, 4894 (1978).