The Semisynthesis of Portions of Hen's-Egg Lysozyme by Fragment Condensation

By A. R. REES and R. E. OFFORD

*Laboratory of Molecular Biophysics, Department of Zoology,
South Parks Road, Oxford OX1 3PS, U.K.*

(Received 23 April 1976)

1. We have evaluated several methods for coupling protected tryptic peptides and have selected one that is satisfactory in terms of efficiency and degree of racemization. 2. We report the use of the chosen method (which must be slightly modified for each application in the light of the result of micro-scale pilot experiments) for the preparation of five fragments of hen's-egg lysozyme ranging in length from 31 to 51 residues. 3. We report methods for the complete deprotection of these fragments. They have been characterized by end-group determination, amino acid analysis and tryptic digestion.

Rees & Offord (1976) have discussed the relative lack of reports of the semisynthesis of proteins and polypeptides by fragment condensation as opposed to stepwise techniques. They described some general methods for the preparation of fragments for condensation, using a limited tryptic digest of hen's-egg lysozyme (EC 3.2.1.17) as the source of material. We report the coupling of some of these fragments to form longer sections of the original enzyme molecule (Fig. 1). We describe for the first time the synthesis of fragments corresponding to residues 1–45, 6–45 and 15–45 of the protein. We have also repeated, on a larger scale and with one change in the protecting groups, the semisyntheses described by Offord (1973) of fragments corresponding to residues 62–113 and 67–113.

Experimental

Materials

Protected fragments of lysozyme (M1, M2, M3, M4, M6, M7 and M8, Fig. 1) were obtained as described by Rees & Offord (1976). 1-Hydroxybenzotriazole (m.p. 157°C) was a gift from Dr. G. T. Young (Dyson Perrins Laboratory, University of Oxford). Dicyclohexylcarbodiimide was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and purified by vacuum-distillation or by discarding ether-insoluble material. The m.p. was above 30°C. Solvents and reagents were obtained in the highest available purity from BDH, Poole, Dorset, U.K. The dimethylformamide (stood over K_2CO_3 , then refluxed with phthalic acid), dimethyl sulphoxide and *N*-ethylmorpholine were redistilled and kept in stoppered containers at 4°C. Volatile decomposition products were removed from the stored dimethylformamide by vacuum-evaporation of a sample to one-third its volume just before use. Any surplus from this procedure was discarded. The stored dimethylformamide

was redistilled each week. The dimethyl sulphoxide used in coupling (but not that used on columns) was kept over molecular-sieve type 3A, 4–8 mesh beads (Fisons, Loughborough, Leics. LB5 9FF, U.K.).

Methods

Pretreatment of peptides. The peptides were treated before coupling to remove traces of carboxylic acid and amine materials that were derived from the buffer systems used at earlier stages in their preparation, and possibly from the atmosphere of the laboratory as well. The treatments also had the effect of converting the α -carboxyl group of the carboxyl component from the carboxylate into the carboxyl form and the α -amino group of the amine component from the ammonium into the amine form. Good coupling yields were normally obtained with materials that had been treated exactly as described below; poor yields often followed even slight departures from the procedure.

Pretreatment of the carboxyl component. The carboxyl component was dissolved or triturated in 2–3 ml of water cooled to 0°C. Trituration of very intractable samples was assisted by dissolving them in the minimum volume of dimethylformamide or dimethyl sulphoxide before adding the water. Sufficient 0.01 M-HCl was then added to bring the pH (externally indicated on pH paper) to about 2.5. The sample was immediately frozen and freeze-dried. (It is essential to avoid vapour-phase contamination from other samples on the freeze-drier.) The dried material is now ready for coupling.

Pretreatment of the amino component. Method A. The peptide was dissolved in dimethylformamide or, if it was insoluble in that solvent, dimethyl sulphoxide and taken to pH 9–10 [externally indicated as described by Rees & Offord (1976)] with triethylamine. If extensive contamination with volatile

Fig. 1. Condensation of the fragments

The sequences of the fragments are taken from Canfield (1963) and Jollès *et al.* (1963). Differences between these two sources were reinvestigated by Rees & Offord (1972). The following abbreviations are used: Boc-NH-CH(CF₃), 1-t-butoxycarbonylamido-2,2,2-trifluoroethyl-; *p*MeOBzl-, *p*-methoxybenzyl-; Mal-, maleyl-; im, unprotected imidazole side chain.

amino compounds is suspected, the pH should be close to 10 and the solution should be taken to dryness and the material redissolved.

Method B. If the peptide was soluble in water, it was dissolved (in 1 ml) and adjusted to pH 10 with aq. (1%, v/v) triethylamine. It was then passed down a column (30 cm $\times$ 1 cm) of Sephadex G-25 in aq. 0.1 M-pyridine and the peptide peak freeze-dried. As before, contamination of samples via the vapour phase must be avoided.

Coupling. Preliminary evaluation of coupling systems. The [Boc-threonine]peptide M7 (Fig. 1) was coupled to Ser-Pro-Phe-Arg-*o*-benzyl [a synthetic

peptide donated (as the HCl salt) by Dr. W. H. Johnson, Hoffmann-LaRoche, Welwyn Garden City, Herts., U.K.]. Four sets of conditions were used.

(1) [Boc-threonine]peptide M7 (4.5 μ mol) was pretreated as above and dissolved in 30 μ l of dimethylformamide. The solution was added to 9 μ mol of the synthetic peptide (solid), and the resulting clear solution cooled to 10°C. Triethylamine (9 μ mol) was added (a precipitate, presumably of the triethylamine hydrochloride, formed), followed by 5 μ mol of *N*-hydroxysuccinimide in 10 μ l of dimethylformamide. The mixture was then cooled to 0°C, and 4.5 μ mol of dicyclohexylcarbodi-imide added in 20 μ l of dimethylformamide. The mixture was then stirred magnetically for 72 h at 4°C.

(2) The same procedure was followed as in step (1), except that 10 μ l of dimethylformamide was substituted for the *N*-hydroxysuccinimide solution.

(3) Boc-peptide M7 (4.5 μ mol) was pretreated and dissolved in 30 μ l of dimethylformamide. Solutions of 1-hydroxybenzotriazole (0.44 M; 8.3 mg in 140 μ l) and of carbodi-imide (0.44 M; 12.6 mg in 140 μ l) were made up. All three solutions were cooled to 0°C. Then 11.25 μ l of the *N*-hydroxysuccinimide and 11.25 μ l of the carbodi-imide solution were added to the peptide, corresponding to molar excesses over peptide of 1.1 for both compounds. After the mixture had been left for 1 h at 0°C and 1 h at 20°C (crystals, presumably of the dicyclohexylurea, were deposited during the second half of this treatment), it was added to 9 μ mol of the synthetic peptide dissolved in the minimum volume of dimethylformamide. The mixture was adjusted to an externally indicated pH of 8.5 with triethylamine (there was a white precipitate) and stirred for 24 h at 20°C.

(4) [Boc-threonine]peptide M7 was converted into the methyl ester with diazomethane in water/methanol (3:17, v/v) as described by Offord *et al.* (1976). The methyl ester was converted into the hydrazide, and 1 μ mol was activated for the azide coupling and allowed to react with 2 μ mol of the synthetic peptide as described by Offord (1972).

The reaction mixtures were each extracted with ether before being dried-down. They were then subjected to preparative paper electrophoresis at pH 1.9 as described (together with all the other paper methods mentioned here) by Gonzalez & Offord (1971). Both coupled product and starting materials (the latter were superimposed at this pH) were eluted. The efficiency of coupling was assessed by amino acid analysis and the degree of racemization by electrophoretic observation of the extent of tryptic digestion (1:125 on a molar basis in pyridine/water, 3:7, v/v; 4 h at 37°C).

Standard method of coupling. The standard method of activation was based on method (3) above. Only small variations were necessary in each case. The correct adjustments to the general conditions were obtained by carrying out trial couplings on an extremely small scale. Although moderate quantities of natural fragments are on the whole easier to obtain than synthetic ones, and the effort does not increase very markedly with length, we regard the use of these very small quantities as essential. It makes possible the exploration of a wide range of several variables without compromising the ability to carry out a bulk coupling afterward. With care, the small-scale results were reproducible and could be applied with confidence to the larger-scale work.

Stage 1. Determination of the correct conditions of activation of the carboxyl component. Pretreated carboxyl component (1 μ mol) was dispensed into a small Durham tube by dissolving the stock sample in methanol (usually 40 μ mol/ml) and taking a measured sample. Both the stock and the sample were dried-down with a gentle stream of N₂. (This operation may have the incidental benefit of removing traces of moisture from the stock.) Dimethylformamide (7 μ l) was added to the tube and the solution cooled to 0°C. Then 2.5 μ l of each of the standard solutions (freshly made up as above) of 1-hydroxybenzotriazole and carbodi-imide were added. Samples (3 μ l) were removed from the mixture at measured intervals, first at 0°C and then at 20°C. Each was immediately expressed from the microcapillary tube on to a Teflon sheet, mixed with 3 μ l of a 16 mm solution of the synthetic peptide in dimethylformamide [previously adjusted to an externally indicated pH of 8.5 (method of Rees & Offord, 1976) with *N*-ethylmorpholine] and then drawn up into the capillary again. The last 3 μ l sample of the activated carboxyl component was mixed with the amino component in the Durham tube rather than being withdrawn. About 0.1 μ l of each mixture was taken to check that the externally indicated pH remained above 8. The capillary was then sealed at both ends with Parafilm and left for 24 h at 20°C. The contents of each capillary were then expressed in two separate lots on to the origin line of a paper chromatogram. Whatman no. 4 paper was used because of its rapid running properties. About

3 μ l of the solution of the synthetic peptide was used as a marker. The chromatogram was developed for a few minutes (distance moved by front = 8 cm) with butanol/acetic acid/water/pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953) and stained with the Sakaguchi reagent for arginine and with Cd/ninhydrin for free amino component. There was usually a middle range of activation conditions in which the characteristic yellow colour given by the amino component had completely disappeared. Longer and shorter times led to there being some staining material left. We take the loss of colour to indicate complete coupling and assume that the poorer results with the longer activation conditions are due to a rearrangement of the active ester to an inactive form. Electrophoresis of over-activated material indicates that the carboxyl groups are still substituted. We always adopt the longest of the satisfactory activation conditions to expose the amino component to the minimum quantity of unchanged carbodi-imide when the amino and carboxyl components are mixed.

The activation conditions were then checked by similar small-scale experiments with a solution of the appropriate natural amino component. The concentration and pretreatment of the amino component was the same as that noted in Table 1 below. Dimethyl sulphoxide was in general a more satisfactory solvent than dimethylformamide. As above, the ratio between carboxyl and amino component was 7:1. The optimum times and temperatures of activation were invariably the same for both the synthetic and the natural amino component.

Finally, a set of trial couplings was carried out with the natural amino component to establish the minimum ratio of the carboxyl component to amino

component required to drive the coupling to completion in 24 h at 20°C. Some couplings were found to require smaller ratios than 7:1 (see Table 1).

Stage 2. Large-scale couplings. Large-scale couplings were carried out under the optimum conditions indicated by the preliminary experiments (details are given in Table 1). The mixtures were stirred magnetically. As a safeguard against accident, the couplings were carried out as two separate operations on half the indicated quantities. The progress of the reaction was followed by paper chromatography of small samples of the coupling mixture as before. As judged by the intensity of the ninhydrin stain, coupling was usually half complete after about 2–3 h and complete after 24 h. The material was then taken to dryness on a freeze-drier and much of the excess of carboxyl component and reagents were extracted with three applications of 1 ml of methanol. The material that remained was then purified by gel filtration on a column (105 cm $\times$ 1.4 cm). Peptides M78, M678 and M34 were separated in the protected state on Sephadex LH-20. Peptides M78 and M678 were run in dimethylformamide, and peptide M34 was run in dimethyl sulphoxide. It was found desirable when using dimethyl sulphoxide to pre-equilibrate the column with freshly distilled eluent. Peptide M234 did not separate from residual peptide M2 on Sephadex LH-20. The peptide was therefore deprotected with trifluoroacetic acid (see below) and run, 10 μ mol at a time, on a column (105 cm $\times$ 1.4 cm) of Sephadex G-25 in aq. 0.025 M-NH₃, brought to pH 10 with solid NH₄HCO₃. The column had been pretreated to prevent adsorption by passing down 1 ml of a 1% solution of bovine serum albumin. A trace of peptide M2 still remained in the M234 peptide and was removed

Table 1. *Individual variations in the bulk couplings*

The activation mixtures of the carboxyl components were 83.3 mM in peptide except for peptide M7, which was 0.1 M. The solvent was always dimethylformamide. Where gel filtration was used to remove carboxyl component from the coupling mixture before deprotection, the solvent was that used for the amino component before coupling.

Peptides coupled	Activation of carboxyl components	Quantity of carboxyl component used (μ mol)	Quantity of amino component used (μ mol) [volume (ml) in parentheses]	Solvent used for amino component	Pretreatment of amino component
M7 to M8	60 min at 0°C followed by 60 min at 20°C	110	15.7 (1.22)	Dimethylformamide	Method B
M6 to M78	60 min at 0°C	30	6 (0.2)	Dimethyl sulphoxide	Method B
M3 to M4	60 min at 0°C followed by 12 min at 20°C	210	50 (2.4)	Dimethyl sulphoxide	Method A
M2 to M34	35 min at 0°C	110	20 (1.3)	Dimethyl sulphoxide	Method A
M1 to M234	60 min at 0°C	60	12 (0.5)	Dimethyl sulphoxide	Method A

as peptide M12 after the next coupling. Peptide M1234 would not separate from peptide M1 and u.v.-absorbing by-products when run in the protected state on Sephadex LH-20. The whole sample was therefore completely deprotected except for the *S*-sulphonato groups (see below). Subsequent trials of gel filtration on Sephadex G-50 and G-25 in aqueous solvents did not remove the u.v.-absorbing material. Ion exchange on CM-Sephadex failed because of the poor solubility of the material at low pH. Chromatography on Sephadex A-25 was only partly successful, as were attempts to precipitate peptide M1234 selectively from solution. Success was only obtained with gel filtration at 45°C on a column (100cm×1.2cm) of Sephadex G-25 in aq. 0.05M-NH₃ adjusted to pH9.0 with solid NH₄HCO₃. The column was pretreated with bovine serum albumin as before. A symmetrical peak of pure peptide was obtained. The u.v.-absorbing impurities (their spectra suggested that they were *p*-methoxy compounds) were eluted in the position taken up by small molecules.

Deprotection. Coupled products were deprotected by exposure to anhydrous trifluoroacetic acid at 4°C (0.2M in redistilled anisole). As the intermediate coupled products were required for physical studies on conformation, all the protecting groups (except the maleyl) were removed at each stage of the synthesis of fragment M1234 (Fig. 1). Peptide M34 was treated for 3h because of the slow deprotection of the *p*-methoxybenzyl group from the aspartate side chain in peptide M3 (Rees & Offord, 1976). Peptides M234 and M1234 were treated for 2h. Peptide M78 was treated for 40min. The tryptophan residue in these fragments appeared, on the basis of u.v. spectroscopy after removal of the maleyl group (see below), to withstand this treatment well. The ratio of the extinction at 280nm to that at 290nm for peptide M34 was found to be 1.6; the ratio for peptide M1234 was 1.4. These values compare with the result of predictions, based on extinction coefficients published for tryptophan and tyrosine by Spande & Witkop (1967) and for oxidized tryptophan by Green (1963), of 1.5 if the tryptophan was untouched and 3.4 if it had been completely oxidized.

Samples of peptide M34 and all of peptide M1234 were treated to remove the maleyl group by dissolving the peptide after trifluoroacetic acid treatment in 2ml of pyridine/acetic acid/water (1:10:89, by vol.) (pH3.5) made 8M in urea (recrystallized from ethanol/water). Neither the formation of cyanate from urea nor the cyanation of amino groups are favoured at this pH. In a trial experiment on peptide M234, the rate of deprotection at 37°C was assessed by reaction of the freed amino groups with 1-fluoro-2,4-dinitrobenzene. The deprotection proceeded with an apparent first-order rate constant of $1.33 \times 10^{-5} \text{ s}^{-1}$, corresponding to a half-time of 14.4h [cf. the value of 11h at 37°C quoted by Butler *et al.* (1969)]. A sample of

peptide M34 and all of M1234 was therefore demaleylated at 37°C for 100h with shaking. The peptides were recovered after deprotection by centrifugation after dilution into 25 vol. of distilled water.

Characterization of products. End groups and amino acid compositions were obtained by the methods described in detail by Gonzalez & Offord (1971). Tryptic digests (enzyme/substrate ratio, 1:100, w/w; 0.5% NH₄HCO₃) were carried out on peptides M34 and M1234 after complete deprotection and (in pyridine/water, 3:7, v/v) on peptides M78 and M678 in the protected state. Peptide M234 was insufficiently soluble for digestion. The digestions lasted for 4h at 37°C, except for that of peptide M1234, duplicates of which were inadvertently allowed to proceed for 6h and for 18h.

Results and Discussion

Following the suggestion by Offord (1969) that peptides from limited tryptic digests could be used in semisynthesis, Izumiya *et al.* (1971) sought optimum conditions for the coupling of synthetic analogues of such tryptic peptides. Our preliminary couplings between a natural and a synthetic fragment (Table 2) show that method (3), which resembles their chosen conditions, is the most satisfactory in that there is no visible racemization and the efficiency of coupling is reasonable. This method appears to have been successfully transferred to the coupling between two natural peptides (Table 3). Peptides that include M4 contain small quantities of isoleucine; the possible reasons are discussed by Rees & Offord (1976). Tryptic digestion of the products appears to be complete, suggesting that there is no significant racemization. The digests normally gave only the starting products. There was a slight trace of the

Table 2. Comparison between different conditions of coupling

Percentage coupled is the percentage of the amount of carboxyl component. The values were not corrected for losses on elution from paper. Such losses are usually of the order of 30%. Percentage racemization in coupled product was a visual estimate of the relative amount of undigested material on a stained electrophoretogram of a tryptic digest.

Conditions (see the text)	Percentage coupled	Percentage racemization in coupled product
1. (Activation <i>in situ</i> ; with hydroxysuccinimide)	57	5-10
2. (Activation <i>in situ</i> ; without hydroxysuccinimide)	50	>20
3. (Prior activation; with 1-hydroxybenzotriazole)	68	0
4. (Azide coupling)	56	0

Table 3. *End groups and compositions of the products*

Predicted ratios are given in parentheses. + indicates that the residue was present but not quantified.

Peptide ...	Amino acid composition (molar ratios)				
	M34	M234	M1234*	M78	M678
Aspartic acid	6.00 (6)	6.00 (6)	6.00 (6)	7.0 (7)	9.00 (9)
Threonine	1.94 (2)	2.06 (2)	2.14 (2)	2.0 (2)	2.16 (2)
Serine	1.81 (2)	2.16 (2)	1.99 (2)	5.8 (6)	5.49 (6)
Glutamic acid	2.04 (2)	3.59 (3)	3.00 (3)		
Proline				1.6 (2)	1.96 (2)
Glycine	3.1 (3)	3.20 (3)	4.02 (4)	2.6 (3)	4.11 (4)
Alanine	2.8 (3)	6.07 (6)	5.54 (6)	5.0 (5)	4.99 (5)
Cysteine	+ (1)	+ (2)	+ (2)	+ (3)	+ (4)
Valine	0.94 (1)	0.86 (1)	1.91 (2)	2.3 (3)	2.64 (3)
Methionine		1.14 (1)	0.88 (1)	0.9 (1)	0.39 (1)
Isoleucine	0.22 (0)	0.27 (0)	0.28 (0)	2.8 (3)	2.64 (3)
Leucine	2.02 (2)	3.11 (3)	3.10 (3)	3.4 (3)	2.64 (3)
Tyrosine	1.91 (2)	1.70 (2)	1.92 (2)		
Phenylalanine	1.61 (2)	1.79 (2)	2.70 (3)		
Tryptophan	+ (1)	+ (1)	+ (1)	+ (2)	+ (4)
Histidine	0.93 (1)	1.37 (1)	0.81 (1)		
Lysine	1.06 (1)	2.16 (2)	3.00 (3)	2.0 (2)	1.86 (2)
Arginine	2.28 (2)	3.19 (3)	3.90 (4)	1.8 (2)	2.93 (3)
End group	His	Cys†	Lys	Thr	—

* 48 h hydrolysis.

† Found as the Dns-Cys(SO₃H) derivative.

deamidated form of [Boc-tryptophan]peptide M6 mono ester [the asparagine residue involved is known to be extremely labile, even when digesting the native protein; see Rees & Offord (1976)]. There were two extra spots in the digest of peptide M1234, the intensities of which increased with the time of digestion and which we believe to be the result of non-tryptic cleavage at residues belonging to peptide M4. Spectroscopic examination (see above) suggests that the tryptophan residue belonging to peptide M4 has escaped serious damage. Nuclear-magnetic-

resonance study of peptides M1234 and M34 (A. R. Rees, J. M. Thornton & R. E. Offord, unpublished work) indicates that the histidine residue belonging to peptide M3 is titrating normally, that the methionine residue belonging to peptide M2 is unharmed and that the protecting groups have been completely removed.

We believe that our methods are generally applicable to fragment-condensation semisynthesis. The particular way in which we have chosen to use them (a large excess of carboxyl component, salt protection

of the carboxyl groups of the amino component and deprotection of side chains at each step) happened to be suitable and convenient for the applications mentioned here. All the carboxyl components were relatively small, so that high concentrations could be used and the excess separated from the coupled product by gel filtration. We deprotected at each step because we wished to carry out physical studies on the intermediates.

Such tactics are not so suitable when both components in the coupling are large. Experience with the resynthesis of the complete chain of cytochrome *c* from a 65- and a 39-residue fragment (C. J. A. Wallace & R. E. Offord, unpublished work) indicates that, although the general methodology is much the same, it is then better to use less disparate quantities of the two components, to protect the carboxyl groups of the amino components covalently and to activate the carboxyl component *in situ*.

We thank the Medical Research Council for financial assistance, and the Oxford City Charities for a grant for A. R. R.

References

- Butler, P. J. G., Harris, J. I., Hartley, B. S. & Leberman, R. (1969) *Biochem. J.* **112**, 679–689
Canfield, R. E. (1963) *J. Biol. Chem.* **238**, 2698–2707
Gonzalez, G. G. & Offord, R. E. (1971) *Biochem. J.* **125**, 309–317
Green, N. M. (1963) *Biochem. J.* **89**, 599–609
Izumiya, N., Noda, K. & Anfinsen, C. B. (1971) *Arch. Biochem. Biophys.* **144**, 237–244
Jollès, J., Jauregin-Adell, J., Bernier, I. & Jollès, P. (1963) *Biochim. Biophys. Acta* **78**, 668–689
Offord, R. E. (1969) *Nature (London)* **211**, 37–40
Offord, R. E. (1972) *Biochem. J.* **129**, 499–501
Offord, R. E. (1973) in *Peptides 1972* (Hanson, H. & Jakubke, H.-D., eds.), pp. 52–56, North-Holland Publishing Co., Amsterdam
Offord, R. E., Storey, H. T., Rees, A. R., Hayward, C. F., Johnson, W. H., Pheasey, M. H. & Wightman, D. A. (1976) *Biochem. J.* **159**, 48–486
Rees, A. R. & Offord, R. E. (1972) *Biochem. J.* **130**, 965–968
Rees, A. R. & Offord, R. E. (1976) *Biochem. J.* **159**, 467–479
Spande, T. F. & Witkop, B. (1967) *Methods Enzymol.* **11**, 498–506
Waley, S. G. & Watson, J. (1953) *Biochem. J.* **55**, 328–337