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The Semisynthesis of Fragments Corresponding to Residues 66–104 of Horse Heart Cytochrome *c*

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We describe the N^{ϵ}-acetimidylation of horse heart cytochrome *c* with retention of biological activity, the cleavage of the modified protein by CNBr, the separation of the fragments, and their further side-chain protection. We describe the manipulation of the amino acid sequences of the fragments by stepwise semisynthetic methods. We have prepared fragments corresponding to residues 66–78 and 66–79 of the protein, as well as the [Asp^{66}] analogue of fragment 66–79. We have prepared the natural sequence and the [*o*-fluoro-Phe^{82}] analogue of the fragment corresponding to residues 81–104 of the protein, and the [N^{ϵ}-trifluoroacetyl-Lys^{79}], the [N^{ϵ}-dinitrophenyl-Lys^{79}] and the [*S*-acetamidomethyl-Cys^{79}] analogues of fragment 79–104, and the [N^{ϵ}-Cbz-Lys^{81}] analogue of fragment 80–104. We have coupled back the fragments of natural sequence to form a semisynthetic fragment corresponding to residues 66–104 of the protein. Modified fragments were also coupled to give analogues of the 66–104-residue sequence. In every case the homoserine residue representing methionine-80 was removed from the *C*-terminus of the 66–80-residue fragment and replaced by methionine on the *N*-terminus of the 81–104 residue fragment during the preparation of the fragments for coupling. The semisynthetic fragments are ready for specific deprotection and further coupling. We have coupled one such fragment to the (1–65)-peptide to produce semisynthetic [Hse^{65}]cytochrome *c*. The product has satisfactory characteristics on chemical analysis, and on assay of its biological activity.

The technique of protein semisynthesis, in which fragments of proteins are used as ready-made intermediates in the chemical synthesis of proteins, has been reviewed as a whole by a number of authors (see Offord & Di Bello, 1978). One approach that appears to offer promise is to use protein fragments produced by CNBr (Offord, 1972). For example, CNBr cleaves horse heart cytochrome *c* at its two methionine residues (65 and 80; Margoliash *et al.*, 1961) to give three fragments corresponding to residues 1–65, 66–80 and 81–104 (which we will call fragments A, B and C respectively). The present paper describes the application of the semisynthetic method to the production of a fragment, and some of its analogues, corresponding to residues 66–104 (which we will call fragment BC). Fragment BC or its analogues can be recombined via a peptide bond with fragment A either by the spontaneous aminolysis of the *C*-terminal homoserine lactone of the latter (Corradin & Harbury, 1974; Barstow *et al.*, 1977; Boon *et al.*, 1978), or by more conventional methods of peptide coupling (Wallace, 1976).

Although cytochrome *c* is of great biological importance, and has been extremely well characterized from a structural point of view, there is no conclusive picture of the mechanism by which it promotes electron transfer. Many of the analogues that would be most useful in elucidating the mechanism are likely to be of low activity, or contain unusual amino acids, and so will not be readily obtained from natural sources. Conventional chemical synthesis of the protein (let alone its analogues) has not proved very easy, and semisynthesis is at present the only feasible route to many of the wanted products. It is noteworthy that fragment B, which is the most easy to modify or replace by a synthetic sequence, contains very many of the residues found to be resistant to evolutionary change and, in particular, residue 80, the site of the sixth ligand to the haem iron.

The strategy employed in the semisyntheses so far undertaken is shown in Fig. 1.

Abbreviations used: Boc, t-butyloxycarbonyl; Cbz, benzyloxycarbonyl; Dns, 5-dimethylaminonaphthalenesulphonyl.

Experimental and Results

Materials

All reagents used in this work, except those listed below, were obtained from BDH, Poole, Dorset BH12 4NN, U.K., and were of the highest obtainable purity.

Horse heart cytochrome *c* (type III), *N*-hydroxysuccinimide, carboxypeptidase A (di-isopropyl

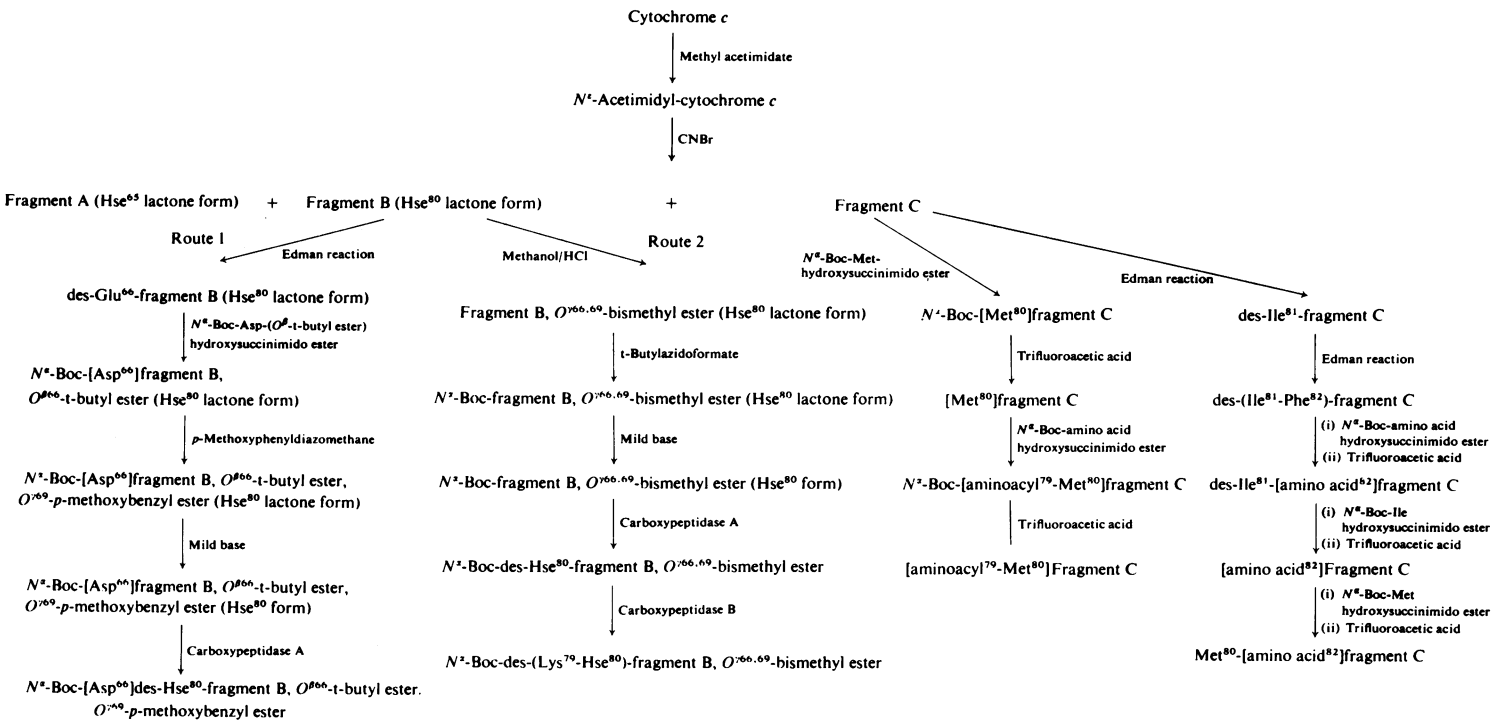

Fig. 1. *Preparation of the fragments*

Fragment A corresponds to residues 1–65 of the intact cytochrome, fragment B to residues 66–80 and fragment C to residues 81–104. For brevity we do not indicate that these fragments, as well as being modified as shown, are all totally N^{ε}-acetimidylated. In addition to the modifications in the Figure, lysine has been inserted in place of isoleucine-81 by a route analogous to that for the substitution at position 82 (see later sections of the text).

phosphorofluoridate-treated), N^{α}-Boc-methionine, N^{α}-Boc-tyrosine, N^{α}-Boc,N^{ε}-Cbz-lysine, N^{α}-Boc-isoleucine, carboxypeptidase B (di-isopropyl phosphorofluoridate-treated) and Tris were obtained from Sigma Chemical Corp., Norbiton Station Yard, Kingston upon Thames, Surrey KT2 7BH, U.K.

Trifluoroacetic acid, phenyl isothiocyanate, racemic *o*-fluorophenylalanine, dicyclohexylcarbodi-imide, N^{α}-Boc-aspartic acid (O^{β}-t-butyl ester), N^{α}-Boc-glutamic acid (O^{γ}-t-butyl ester) and t-butylazidoformate were obtained from Koch–Light Laboratories, Colnbrook, Bucks. SL3 0BZ, U.K.

3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine and dimethyl sulphoxide were obtained from Fluka A.G., through their U.K. agents, Fluorochem Ltd., Glossop, Derbyshire SK13 9NU, U.K.

CNBr came from Eastman–Kodak, through their U.K. agents, Kodak Ltd., Kirkby, Liverpool L33 7UF, U.K.

1-Hydroxybenzotriazole and N^{α}-Boc-(*S*-acetamidomethyl)cysteine were gifts from Dr. G. T. Young of the Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY, U.K.

N^{α}-Boc-*o*-fluoro-L-phenylalanine was prepared from the racemic form of the parent fluorophenylalanine as described by Saunders & Offord (1977); N^{α}-Boc,N^{ε}-trifluoroacetyl-lysine was prepared from N^{α}-Boc,N^{ε}-Cbz-lysine by catalytic hydrogenation to free the ε-NH_2 group, followed by reaction with *S*-ethyl thiotrifluoroacetate (Schallenburg & Calvin, 1955).

Methods

Amino-group protection of cytochrome c. The acetimidyl protecting group of Hunter & Ludwig (1962) was employed for amino-group protection by a modification of their method. Methyl acetimidate hydrochloride (0.66g) (prepared by the method of Pinner, 1892) in 0.8ml of 5M-NaOH was added to 100mg of cytochrome *c* in 10ml of 0.1M-$Na_2B_4O_7$ solution and left at 0°C for 30min. It was then allowed to reach room temperature (20°C). The pH was adjusted to 9.5 and the mixture left at 20°C for 1h. Then 0.33g of reagent in 0.27ml of 5M-NaOH was added and the pH re-adjusted to 9.5. After 1h at 20°C this latter procedure was repeated. Finally 0.66g of reagent in 0.6ml of 5M-NaOH was added and the mixture left at 4°C overnight.

Protected protein was separated from unwanted compounds by repeated dialysis, and then freeze-dried. The extent of protection was judged by the trinitrobenzenesulphonic acid assay of Fields (1971). In this assay 0.87mM-cytochrome *c* gives a colour intensity of 0.163 absorbance unit at 420nm. The product of the acetimidylation reaction gave an intensity of 0.000. Alternatively, the degree of acetimidylation can be determined by extrapolation back to zero time of the yields of lysine and its acetimidyl derivative obtained during a time course of acid hydrolysis. Samples of protein were hydrolysed and analysed in the usual way (e.g. Gonzalez & Offord, 1971), and the quantity of *N*-acetimidyl-lysine was determined. This molecule, which has an analyser constant of 0.85 that of lysine (Wallace, 1976), is slowly converted back into lysine. Such a time course gave a zero-time value corresponding to 96% protection. We conclude from these results that effectively complete protection is readily obtained.

A valuable feature of this protecting group is illustrated by the fact that the fully protected protein exhibits 90–100% of the activity of native cytochrome *c* in the depleted-mitochondria assay of Jacobs & Sanadi (1960) and after deprotection [effected by treatment with a conc. ammonium acetate buffer, pH 11.3, for 24h at room temperature (Ludwig & Byrne, 1962)] the product exhibits 90% activity (Fig. 2). These observations were not unexpected in view of the rather small difference that the group makes to the properties of the lysine side chain, and in the light of the experience of previous authors, who have used it in semisynthetic work with other proteins (Webster & Offord, 1972; Webster, 1972; Slotboom & De Haas, 1975).

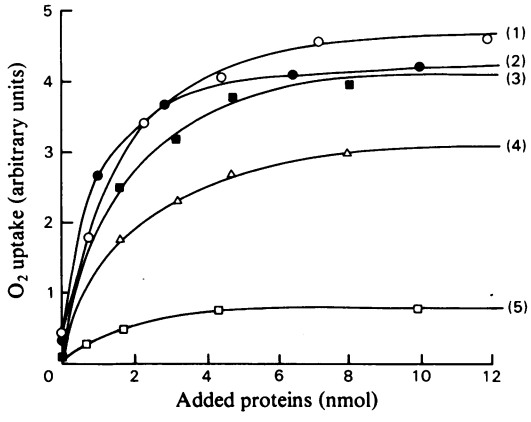

Fig. 2. *Stimulation of oxygen uptake by cytochrome c-depleted mitochondria by various cytochrome c derivatives*

The assay procedure was exactly as described by Harris & Offord (1977). (1) Native cytochrome *c*. (2) Acetimidylated cytochrome *c*. (3) Deprotected acetimidylated methylated cytochrome *c*. (4) Acetimidylated cytochrome *c* resynthesized from naturally obtained fragments. In this instance only, the CNBr cleavage was carried out according to the conditions of Corradin & Harbury (1974), under which there is a good recovery of residue 80 in its intact methionine form. (5) Acetimidylated methylated cytochrome *c*.

Table 1. *Amino acid compositions and end groups of CNBr fragments*

Hydrolyses for the analyses recorded in this and subsequent Tables were carried out at 108°C in 6M-HCl/phenol (100:1, w/v), typically for 18–24h. The predicted values are based on the sequence (Margoliash *et al.*, 1961). Homoserine was not determined. Where no value is given for any other amino acid in this and other Tables, it was present in amounts less than the equivalent of a molar ratio of 0.1. n.d., Not determined.

	Residues 1–104			Residues 1–80 (fragment AB)		Residues 1–65 (fragment A)		Residues 66–104 (fragment BC)		Residues 81–104 (fragment C)		Residues 66–80 (fragment B)	
		Observed											
	Predicted	$(ABC)_n$	ABC	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed
Amino acid													
Aspartic acid	8	7.8	8.0	6	6.2	5	5.0	3	2.9	2	2.1	1	0.97
Threonine	10	9.5	9.5	8	7.6	7	6.9	3	2.8	2	2.0	1	1.0
Glutamic acid	12	12.5	12.4	9	9.0	7	7.3	5	5.1	3	3.1	2	2.1
Proline	4	3.2	4.4	4	3.4	2	1.6	2	2.1	—	—	2	2.0
Glycine	12	12.6	12.4	11	10.8	10	9.9	2	2.5	1	1.0	1	0.96
Alanine	6	5.6	6.1	3	3.5	3	3.3	3	3.0	3	3.0	—	—
Cysteine	2	n.d.	n.d.	2	n.d.	2	n.d.	—	—	—	—	—	—
Valine	3	2.3	2.8	3	2.8	3	2.9	—	—	—	—	—	—
Methionine	2	—	—	—	—	—	—	—	—	—	—	—	—
Isoleucine	6	5.0	4.4	3	2.8	2	1.8	4	3.0	3	2.0	1	0.96
Leucine	6	5.7	5.6	4	4.0	3	3.0	3	3.1	2	1.7	1	1.1
Tyrosine	4	2.8	2.8	3	1.9	1	1.1	3	2.7	1	0.96	2	1.7
Phenylalanine	4	3.7	3.5	3	2.7	3	2.5	1	0.8	1	0.65	—	—
Histidine	3	3.2	3.5	3	3.1	3	2.7	—	—	—	—	—	—
Lysine*	19	19.6	19.4	14	14.6	11	11.4	8	8.4	5	4.9	3	3.2
Arginine	2	2.0	2.0	1	1.1	1	1.0	1	1.0	1	1.0	—	—
End group†	—	None	None	—	None	—	None	Glu	Glu	Ile	Ile	Glu	Glu

* In this and the other Tables this is lysine + N^{ϵ}-acetimidyl-lysine.

† The *N*-terminus of cytochrome *c* is *N*-acetylglycine (Margoliash *et al.*, 1961).

Cleavage of the protein and fragment separation. The cleavage of cytochrome *c* by CNBr has been studied by Black & Leaf (1965) and by Corradin & Harbury (1970). When these workers' conditions are used on acetimidyl-cytochrome *c*, the fragmentation pattern is very similar to that of the native protein.

For the purposes of this work, slightly different conditions were employed, which trial experiments had shown would result in different proportions of the seven possible products from a cleavage mixture. (These products are fragments A, B and C, together with the fragments corresponding to cleavage at only one methionine residue, both with and without conversion of the uncleaved residue to homoserine. We do not count the lactone and open-ring forms of a peptide separately. For brevity, we will normally write the names of fragments A, B and C without an indication of the fact that, throughout these experiments, they are totally N^{ε}-acetimidylated.) An 120-fold excess of CNBr over methionine residues was employed in a 0.8mM solution of acetimidyl-cytochrome *c* in 0.1M-HCl. The reaction was performed at 20°C for 24h, and the mixture was then freeze-dried.

The freeze-dried digest mixture was redissolved in 7% formic acid, and subjected to gel filtration on a column (135cm × 2cm) of Sephadex G-50 (fine grade). A typical elution profile is shown in Fig. 3. The fractions were identified by their amino acid compositions and end groups (Table 1); they were freeze-dried and kept stored at −20°C before use. Table 1 shows that the fragments are quite pure, but in any case they receive additional purification as a part of subsequent operations.

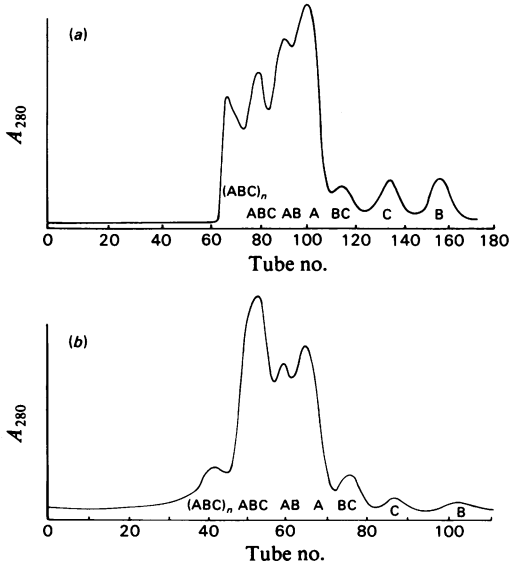

Fig. 3. *Elution profiles from gel filtration of CNBr digests of horse heart cytochrome c on Sephadex G-50 (fine grade)*
(*a*) 120-fold molar excess of CNBr over protein in 0.1M-HCl; (*b*) 3-fold molar excess of CNBr over protein in 70% formic acid. Column dimensions were 2cm × 135cm, and the eluent was 7% formic acid.

Modifications to fragment B. When we first attempted to couple fragment A to purified samples of fragment BC in active-ester and azide couplings (C. J. A. Wallace & R. E. Offord, unpublished observations), little or no coupling occurred. This we found to be the result of conversion of the *N*-terminal glutamic residue into pyrrolidonecarboxylic acid. The problem was avoided by the immediate amino-group protection of fragment B or by the substitution of aspartic acid for its *N*-terminal glutamic acid by means of the following sequences of reactions.

(*a*) Route 1: replacement of glutamic acid-66. Fragment B was allowed to react at 37°C for 150min with a 1000-fold excess of phenyl isothiocyanate in water/pyridine (2:3, v/v). After removal of the solvent and reagent on the freeze-drier, the *N*-terminal residue was cyclized off with anhydrous trifluoroacetic acid. Dansylation of the purified product, des-Glu^{66}-fragment B (called fragment $_{-1}$B in the Tables below), showed the only end group to be tyrosine. Amino acid analysis, however, showed removal of glutamic acid to be less than quantitative (Table 3). We take this to be due to the presence of material in which glutamic acid had been converted to pyrrolidonecarboxylic acid before the Edman reaction was undertaken. The proportion of residual glutamic acid increased with the amount of prior handling the fragment had received.

N^{α}-Boc-aspartic acid (O^{β}-t-butyl ester) *N*-hydroxysuccinimido ester was added as the solid in 10-fold molar excess to 1mM-des-Glu^{66}-fragment B in dimethylformamide, with the simultaneous addition of 1-hydroxybenzotriazole, also in 10-fold molar excess over the peptide (König & Geiger, 1972). After 24h at 20°C, the peptide was separated from excess reagents by gel filtration on Sephadex LH20 in dimethylformamide/water (17:3, v/v). Column dimensions suitable for a 50mg sample of peptide were 2.5cm diam. × 100cm high. The flow rate was 50ml/h. Complete reaction, as judged by electrophoretic mobility, loss of tyrosine as the peptide's end group on dansylation, and amino acid analysis (Tables 2 and 3), was obtained in 24h at 20°C. Aspartic acid replaced tyrosine as the end group of the deprotected peptide, but, as shown in Table 3, aspartic acid and glutamic acid do not give integral values. This results from the partial conversion of glutamic acid-66 into pyrrolidonecarboxylic acid before it can be removed by the Edman reaction. Only the fraction that escapes conversion is replaced by aspartic acid, and the remainder reverts to glutamic acid on acid hydrolysis. Although wasteful, this side reaction is not fatal. The by-product could be removed after N^{α}-deprotection of the true product or (as we prefer)

Table 2. *Properties of derivatives of fragment B*

Staining methods were as described by Offord (1969). Mobilities were measured and predicted as described by Offord (1966).

Derivative	Mobilities at pH 6.5			Staining properties	
	Observed	Predicted	Charge	With ninhydrin	For tyrosine
Unmodified fragment B lactone	0.320	0.330	+2	+	+
Unmodified fragment B open ring	0.170	0.175	+1	+	+
Fragment B ($O^{\gamma 66,69}$-bismethyl ester) lactone	0.620	0.650	+4	+	+
N^{α}-Boc-fragment B ($O^{\gamma 66,69}$-bismethyl ester) lactone	0.480	0.485	+3	−	+
N^{α}-Boc-fragment B ($O^{\gamma 66,69}$-bismethyl ester) open ring	0.330	0.330	+2	−	+
N^{α}-Boc-fragment B ($O^{\gamma 66}$-methyl ester) open ring	0.170	0.175	+1	−	+
N^{α}-Boc-fragment B_{-1} ($O^{\gamma 66,69}$-bismethyl ester)	0.375	0.340	+2	−	+
N^{α}-Boc-fragment B_{-2} ($O^{\gamma 66,69}$-bismethyl ester)	0.202	0.195	+1	−	+
Fragment $_{-1}$B lactone	0.480	0.505	+3	+	+
Fragment $_{-1}$B open ring	0.340	0.340	+2	+	+
N^{α}-Boc-[Asp^{66}]fragment B ($O^{\beta 66}$-t-butyl ester) lactone	0.320	0.305	+2	−	+
N^{α}-Boc-[Asp^{66}]fragment B ($O^{\beta 66}$-t-butyl ester) open ring	0.180	0.160	+1	−	+
[Asp^{66}]Fragment B lactone	0.330	0.330	+2	+	+
[Asp^{66}]Fragment B open ring	0.160	0.175	+1	+	+
N^{α}-Boc-[Asp^{66}]fragment B ($O^{\beta 66}$-t-butyl ester, $O^{\beta 69}$-*p*-methoxybenzyl ester) lactone	0.460 (streaks)	0.450	+3	−	+
N^{α}-Boc-[Asp^{66}]fragment B ($O^{\beta 66}$-t-butyl ester, $O^{\beta 69}$-*p*-methoxybenzyl ester) open ring	0.290 (streaks)	0.300	+2	−	+
N^{α}-Boc-[Asp^{66}]fragment B_{-1} ($O^{\beta 66}$-t-butyl ester, $O^{\beta 69}$-*p*-methoxybenzyl ester)	0.315	0.310	+2	−	+

Table 3. *Amino acid compositions and end groups of derivatives of fragment B*

The predicted values were based on the sequence (Margoliash *et al.*, 1961). Where no value is given for an amino acid, it was present in amounts less than the equivalent of a molar ratio of 0.1. When homoserine was determined, the method of Ambler (1965) was used. n.d., Not determined. The meaning of the symbols $_{-1}$B, B_{-1}, B_{-2} is given in the text.

Amino acid	Fragment B (predicted)	Fragment B	Fragment $_{-1}$B	[Asp^{66}]-Fragment B	[Asp^{66}]-Fragment B_{-1}	Fragment B_{-1}	Fragment B_{-2}
Aspartic acid	1	0.97	0.89	1.5*	1.5*	0.96	0.93
Threonine	1	1.0	0.87	1.0	1.0	0.77	0.76
Homoserine	1	n.d.	n.d.	1.0	0.09	n.d.	n.d.
Glutamic acid	2	2.1	1.2*	1.5*	1.4*	2.0	1.9
Proline	2	2.0	1.9	1.5	2.1	1.9	1.7
Glycine	1	0.96	0.91	1.1	1.2	1.2	1.1
Isoleucine	1	0.96	0.90	0.79	0.96	0.89	0.93
Leucine	1	1.1	1.0	0.88	0.94	0.99	1.0
Tyrosine	2	1.7	1.7	1.5	1.6	1.6	1.7
Lysine	3	3.2	3.1	3.0	3.2	3.0	2.1
N-Terminal group†	Glu	Glu	Ψyr	Asp	Asp	Glu	Glu

* The non-integral values for Asp and Glu are discussed in the text.

† No other *N*-terminal groups seen. *O*-Dns-Tyr seen.

allowed to remain; it cannot participate in coupling reactions as an amino component, and is automatically eliminated when the coupling mixture is worked up.

(*b*) Route 1: carboxy-group protection of fragment B. The ability of the *C*-terminal homoserine to lactonize is one of the advantages of CNBr fragments as semisynthetic intermediates, as this property provides a means of differentiating between side-chain and terminal carboxyl groups (Offord, 1972). The fragments are released in the lactone form, and any material that is converted to the open form on handling largely reverts to the lactone during the cyclization step of the Edman degradation. Even after the further handling described above, 80% of fragment B is in the lactone form, as judged by its electrophoretic mobility at pH6.5 (see Table 2). We chose to use *p*-methoxyphenyldiazomethane as the esterifying agent. The reagent was prepared as described by Offord *et al.* (1976), and added dropwise as an ethereal solution (approx. 0.1M) to a solution (1mM) of peptide in dimethylformamide/water (17:3, v/v), over the course of 1h. The reaction vessel was kept at 0°C and very vigorously stirred. The pH was maintained at 4.5 by the delivery of 0.1M-HCl from a pH-stat. A series of trial experiments, in which esterification was followed by the change in mobility on paper electrophoresis at pH6.5, showed that a 100-fold molar excess of diazoalkane over carboxy groups was necessary for complete esterification. The reaction mixture was stirred for 1h at 0°C after the addition of diazoalkane was finished. The pH was then adjusted to 7.0 with 0.1M-NaOH. The ether layer was then removed, the dimethylformamide layer extracted twice with an equal volume of diethyl ether, and then dried under a high vacuum. The residue was subjected to gel filtration on the column of Sephadex LH20 in dimethylformamide/water (17:3, v/v) as described above.

(*c*) Route 2: protection of side-chain carboxy groups. Semisynthetic derivatives of fragment B that, unlike the [N^{α}-Boc,O^{β}-t-butyl-Asp^{66}] derivative, do not already have acid-labile protecting groups, can be made from fragments esterified directly with methanolic HCl by the method of Chibnall *et al.* (1958). The peptide was dissolved (5mg/ml) in anhydrous methanol made 0.1M in HCl; the solution was left for 24h at 20°C before drying by rotary evaporation. These strongly acidic conditions favour closure of the lactone, so that the proportion of material esterified at the *C*-terminus is very small, and side-chain esterification is complete (Table 2).

(*d*) Route 2: protection of the α-amino group. A 500-fold molar excess of t-butylazidoformate was added to a 10mM solution of the peptide ester in dimethyl sulphoxide, and the pH [externally indicated on wet pH paper as described by Rees & Offord (1976*a*)] adjusted to 8.5. The mixture was left at 20°C for 24h, then dried under high vacuum. Paper electrophoresis showed that reaction was complete (Table 2).

(*e*) Routes 1 and 2: opening the lactone ring. The conditions required for the opening of the ring of homoserine lactone in peptides vary from peptide to peptide (Ambler, 1965). We therefore tested the efficiency of a number of alkaline buffers, between pH8.0 and 10.5, on the lactone form of fragment B. The temperature was 20°C. We observed that all conditions tested caused some degree of loss of the *p*-methoxybenzyl group from glutamic acid-69. The extent of the loss (as judged by staining intensities on paper electrophoretograms) ranged from about 10% at pH8 to 100% at pH10.5. This unexpected lability of the *p*-methoxybenzyl group may, in part, be due to the environment of the residue itself, for it was found that methyl-group protection also exhibited unusual lability (though not to quite such a degree; see below) when applied to this residue. We are able to suggest that the base-labile group is on residue 69 and not on residue 66, at least when the material is that prepared by route 1. Here, the side-chain carboxy group of residue 66 is protected by the t-butyl group, which is stable to alkali.

We chose pH9.0 for the ring opening [the reaction took place in a 2% (w/v) solution of redistilled triethylamine, adjusted to pH9 (at 20°C) with CO_2, 1mM in peptide]. Under these conditions the process went to completion within a reasonable period (2h) at 20°C. About 25% of the material had also lost a side-chain ester by this time. If the methyl group is used for esterification, this proportion falls to about 10%. The loss of the *p*-methoxybenzyl group is quite large, but was judged to be acceptable because we found that the monoesterified derivative was resolved from the diesterified derivative on the column of Sephadex LH20 described above. In practice, this operation was deferred until after the next step; for the time being the reaction mixture was simply adjusted to neutrality with 1M-acetic acid and the solution freeze-dried.

(*f*) Routes 1 and 2: stepwise degradation from the *C*-terminus. The removal of *C*-terminal amino acids from fragment B was achieved by using carboxypeptidases. The *C*-terminal homoserine was removed with carboxypeptidase A. With an enzyme/substrate ratio of 1:100 (molar proportions), in a 0.1M-*N*-ethylmorpholine/acetate buffer, pH7.5, the cleavage goes to completion in 2h at 20°C (Table 3). The peptide concentration generally employed was 5mM; the appropriate amount of suspension of carboxypeptidase A was first brought into solution in 2M-NH_4HCO_3. The reaction was stopped by the addition of 4vol. of dimethylformamide; the mixture is then applied directly to the Sephadex LH20 column described above. The major peptide peak is the diester (Fig. 4).

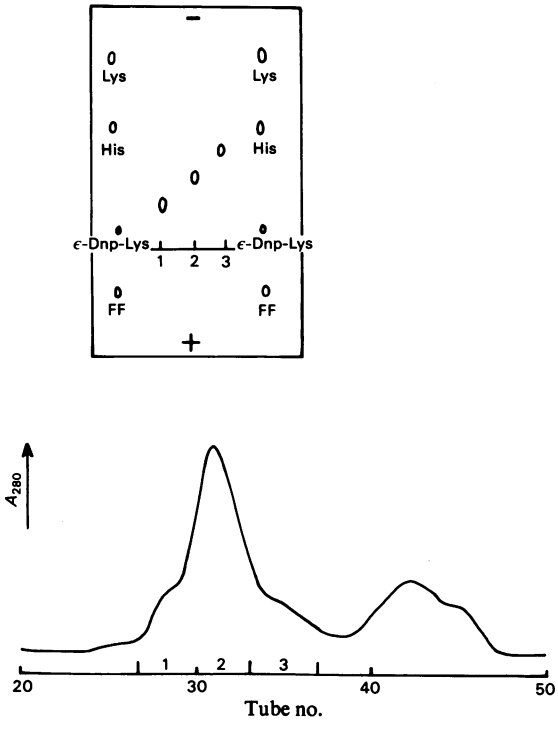

Fig. 4. *Elution profile of the separation on Sephadex LH20 of products from the carboxypeptidase A digestion of modified fragment B*

Experimental details are given in the text. The three fractions were electrophoresed on paper at pH6.5 and the result is shown in the inset. 1 is the monomethyl derivative (charge +1), 2 is the dimethyl (charge +2), and 3 is the dimethyl derivative with the homoserine lactone ring closed (charge +3). Abbreviations: Dnp, dinitrophenyl; FF, Xylene Cyanol FF.

The progress of degradation can be monitored by paper electrophoresis of samples of the reaction mixture at pH1.9. [This and all other analytical methods were carried out as described by Gonzalez & Offord (1971).] We found no evidence for the release of N^{ε}-acetimidyl-lysine (the penultimate residue) from the peptide. Our hope, in choosing the acetimidyl group, that the modified lysine residue would resemble arginine rather than lysine in its resistance to carboxypeptidase A, was therefore justified.

However, also as hoped, carboxypeptidase B was found to remove N^{ε}-acetimidyl-lysine from the *C*-terminus of des-homoserine-fragment B, with no accompanying loss of threonine-78. The conditions of digestion followed those of Ambler (1967). We found that a 1:500 molar ratio of enzyme to substrate was sufficient for complete removal within 1h at 20°C when the substrate was 4mM in a 0.025M-Tris/0.1M-NaCl buffer adjusted to pH7.65 at 20°C with 1M-HCl. Again, we followed the reaction by paper electrophoresis of samples at pH1.9.

The reaction mixture (generally 5ml) was diluted with 15ml of dimethylformamide after 1h and applied to a column of Sephadex LH20 in dimethylformamide/water (17:3, v/v). The major peak of the elution profile is the desired derivative (Fig. 4, Table 3).

By the use of these two routes, three derivatives of fragment B were prepared for coupling: (1) N^{α}-Boc, $N^{\varepsilon 72,73}$-bisacetimidyl, des-(Lys^{79}-Hse^{80})-fragment B, $O^{\gamma 66,69}$-bismethyl ester; (2) N^{α}-Boc, $N^{\varepsilon 72,73,79}$-trisacetimidyl, des-Hse^{80}-fragment B, $O^{\alpha 66,69}$-bismethyl ester; (3) N^{α}-Boc, $N^{\varepsilon 72,73,79}$-trisacetimidyl, des-Hse^{80}-[Asp^{66}]fragment B, $O^{\beta 66}$ t-butyl ester, $O^{\gamma 69}$-*p*-methoxybenzyl ester. These three products will be called fragments B_{-2}, B_{-1} and B'_{-1} respectively.

Modifications to fragment C. (*a*) Edman degradation. Two cycles of the Edman reaction were used to remove the *N*-terminal residues isoleucine-81 and phenylalanine-82 from the fragment. The procedure adopted was essentially the same as that used for degradation of fragment B. Trials with 100-, 200- and 500-fold molar excesses of phenyl isothiocyanate showed that a 500-fold excess was necessary for quantitative removal of such residues (Table 4). After two cycles, the degraded peptide was purified by gel filtration on Sephadex G-50 in 7% formic acid.

(*b*) Replacement of the *N*-terminal residues and extension of fragment C. Stepwise condensation to the terminus of fragment C and its truncated derivatives was achieved by the use of Boc-amino acid *N*-hydroxysuccinimido esters. We prepared the active esters listed in Table 5 by the methods described by Saunders & Offord (1977).

Coupling was performed in dimethylformamide solution, at a peptide concentration of 1mM, by using a 10–20-fold molar excess of active ester and an equal excess of 1-hydroxybenzotriazole (König & Geiger, 1972). Reaction was allowed to continue for 24h at 20°C. Under these conditions coupling always appeared to be quantitative (Table 4). The peptide product was separated from small molecules by gel filtration on Sephadex LH20 as usual. Once dried under high vacuum the peptide could be treated with anhydrous trifluoroacetic acid (4°C for 60min, peptide concentration 5mM) to remove the α-amino-group protection. The acid was removed under high vacuum and a further purification step, gel filtration on Sephadex G-50 in 7% formic acid (column dimensions 2cm diam. × 135cm high; flow rate 40ml/h) generally followed.

The determination of *o*-fluorophenylalanine in these fragments by amino acid analysis is unsatisfactory, even if a range of hydrolysis times is used. Tyrosine, with which *o*-fluorophenylalanine emerges from the analyser column, is subject to slow destruction during hydrolysis and, in the fragments described

Table 4. *Amino acid compositions and end groups of derivatives of fragment C*

The predicted values are based on the sequence (Margoliash *et al.*, 1961). Amino acids not represented in the Table were present in amounts less than the equivalent of a molar ratio of 0.1. n.d., Not determined.

Amino acid	Fragment C (predicted)	Fragment C	Fragment $_{-1}$C	Fragment $_{-2}$C	[FPhe^{82}]-Fragment $_{-1}$C	[FPhe^{82}]-Fragment $_{+1}$C	Fragment $_{+1}$C	[N^{ϵ}-dinitrophenyl-Lys^{79}]-Fragment $_{+2}$C	[N^{ϵ}-trifluoroacetyl-Lys^{79}]-Fragment $_{+2}$C	[Cys^{79}]-Fragment $_{+2}$C	[N^{ϵ}-Cbz-Lys^{81}]-Fragment $_{+1}$C
Aspartic acid	2	2.1	2.0	2.0	2.0	2.2	2.0	2.1	2.0	2.0	2.0
Threonine	2	2.0	1.9	2.0	1.8	1.9	1.9	2.0	2.1	1.8	1.7
Glutamic acid	3	3.1	3.1	3.0	3.0	3.1	2.8	3.2	2.9	3.0	3.0
Glycine	1	1.0	1.0	1.2	1.2	1.3	0.93	1.1	1.2	n.d.*	1.2
Alanine	3	3.0	3.0	2.8	2.8	2.9	3.0	3.1	3.1	n.d.*	2.7
Cysteine	—	—	—	—	—	—	—	—	—	0.94	—
Methionine	—	—	—	—	—	0.55†	1.0	0.91	0.30†	Present†	0.68
Isoleucine	3	2.0	1.1	1.0	0.81	1.6	2.0	2.0	2.1	1.9	1.4
Leucine‡	2	1.7	1.7	1.7	1.7	1.8	1.9	2.0	1.9	1.9	1.9
Tyrosine	1	0.96	0.86	0.90	1.6§	1.6§	0.94	1.0	0.94	0.96	0.92
Phenylalanine‡	1	0.65	0.90	0.06	—	—	0.51	0.74	0.89	0.91	0.88
Lysine	5	4.9	5.0	4.9	4.8	5.0	5.0	5.0	6.0	n.d.*	6.0
N^{ϵ}-Dinitrophenyl-lysine	—	--	—	—	—	—	—	0.88‖	—	—	—
Arginine	1	0.98	0.90	1.0	0.89	1.0	0.94	0.94	0.94	n.d.*	0.94
N-Terminal group¶	Ile	Ile	Phe	Ala	FPhe	Met	Met	n.d.	Lys**	Cys	Met

* Analyser failure.

† Thioglycollic acid not included in the hydrolysis mixture.

‡ Leucine and phenylalanine are next to a slowly released residue (isoleucine in both cases).

§ *o*-Fluorophenylalanine runs with tyrosine. Like the phenylalanine that it replaces, the fluorophenylalanine will be slowly liberated on hydrolysis owing to its neighbouring isoleucine.

‖ An average analyser constant (0.96 that of aspartic acid) was assumed.

¶ The end group given is the major one; very minor traces of contamination with other amino acids were sometimes seen. When isoleucine was the end group, another bright spot (probably Dns-Ile-Phe) was seen. *O*-Dns-Tyr was usually observed.

** Observed as N^{α}-Dns-Lys.

Table 5. *Characterization of amino acid derivatives*

Sources of values: (1) Anderson *et al.* (1964); (2a) Fletcher & Jones (1972); (2b) Fletcher & Jones (1975); (3) Saunders & Offord (1977); (4) Dr. G. T. Young, Dyson Perrins Laboratory, Oxford, personal communication; (5) Saunders (1974). Where no source is indicated, we believe the compound in question to be a new one.

Derivative	Recrystallization solvent	Melting point (°C) Experimental (uncorrected)	Melting point (°C) Literature value	Source
N^{α}-Boc-Asp (O^{β}-t-butyl ester) *N*-hydroxysuccinimido ester	Propan-2-ol	119–121		
N^{α}-Boc-Glu (O^{γ}-t-butyl ester) *N*-hydroxysuccinimido ester	Propan-2-ol	138–139		
N^{α}-Boc-Met *N*-hydroxysuccinimido ester	Propan-2-ol	132–133	128–129	1
N^{α}-Boc-Ile *N*-hydroxysuccinimido ester	Diethyl ether/light petroleum (b.p. 60–80°C)	74–76	92–93 101–103 from di-iso-propyl ether	1 2a
N^{α}-Boc-*o*-F-Phe *N*-hydroxysuccinimido ester	Propan-2-ol	158–163	158–159	3
N^{α}-Boc,N^{ϵ}-dinitrophenyl-Lys *N*-hydroxysuccinimido ester	Propan-2-ol	76–78		
N^{α}-Boc-*S*-acetamidomethyl-Cys trichlorophenyl ester	Ethyl acetate/light petroleum (b.p. 60–80°C)	134–135		4
N^{α}-Boc,N^{ϵ}-trifluoroacetyl-Lys *N*-hydroxy-succinimido ester	Ethyl acetate/light petroleum (b.p. 60–80°C)	Oil	118	2b
N^{α}-Boc,N^{ϵ}-Cbz-Lys *N*-hydroxysuccinimido ester	Ethyl acetate/light petroleum (b.p. 60–80°C)	79–82		
N^{α}-Boc-Tyr *N*-hydroxysuccinimido ester	Propan-2-ol	200–203	175–177 180–182 from ethyl acetate/light petroleum (3:4, v/v)	5 2a

in this paper, shares with the fluorophenylalanine residue a degree of slow release due to hindrance of hydrolysis by neighbouring residues. End-group analysis at the appropriate stage provides useful supporting evidence that successful substitution has been achieved, but is only semi-quantitative at best. We place most reliance on spectroscopic examination of the final product. Thus, the absorption spectrum of the purified [*o*-F-Phe^{82}]fragment BC (Fig. 5, Table 6, below) has two fused peaks at 262nm and 268nm, which are expected of *o*-fluorophenylalanine (Saunders, 1974) and are not seen in the materials that lack this amino acid. If allowance is made for the absorbance at 262nm expected from the three tyrosine residues in the fragment (Mihahyi, 1969), the residual absorbance at 262nm of a 0.2mM solution of the fragment is 0.156cm^{-1}. This, based on a ε_{262} value for the *o*-fluorophenylalanine of 840M$^{-1}\cdot$cm^{-1} (Saunders, 1974), corresponds to a content of the amino acid of 0.93mol/mol of fragment.

The above techniques were used both to effect the substitution of residues within the sequence of fragment C and to extend the sequence at the *N*-terminus (see Fig. 1). The derivatives synthesized, and their amino acid compositions, are shown in Table 4. The notation $_{+1}$C, by analogy with that used for the fragment-B series, indicates the extension of the fragment by one residue at the *N*-terminus.

Synthesis of derivatives of fragment BC. The derivatives of the two CNBr fragments, B and C, that we have prepared were employed in fragment-condensation couplings to prepare semisynthetic samples of fragment BC. Methods for efficiently performing such couplings in the case of tryptic peptides of hen's-egg lysozyme have been described (Rees & Offord, 1976*b*), and their best method (pre-activation of the carboxyl component to the 1-hydroxybenzotriazole ester, followed by addition to the amino component) was used in this case. Rees & Offord (1976*b*) reported that the esters formed are not stable, and decay to an inactive, though still esterified, form. When using this method it is necessary to determine an optimum activation time, at which the best compromise between ester synthesis and inactivation is achieved; this time depends on the peptide employed and is quite variable.

The optimum conditions were found by a series of very-small-scale trial couplings to a synthetic tetrapeptide Ser-Pro-Phe-Arg(NO_2) benzyl ester, as described by Rees & Offord (1976*b*). In general, portions of activation mixtures were removed at intervals of 15, 30 and 60min at 0°C, and at 60min at 0°C plus 30 and 60min at 20°C. These samples were then employed in coupling mixtures. In the case of fragment B_{-1}, those removed at 30 and 60min at 0°C gave highest coupling efficiencies, whereas for fragment B_{-2}, the optima were 60min at 0°C and 60min at 0°C plus 30min at 20°C. Before preparative couplings were attempted, the chosen conditions were checked on a small scale by the use of fragment C. In both the trial and the preparative couplings the carboxyl components (e.g. B_{-1} or B_{-2}) were pretreated by freeze-drying from a 2mM solution in 5mM-HCl, followed by gel filtration on Sephadex LH20 in methanol. The peptide peak was dried by a stream of N_2, freeze-dried again from 5mM-HCl, dissolved (10mM) in anhydrous redistilled methanol and dried, first under a stream of N_2 and then under high vacuum. Preparations of fragment-C derivatives were pretreated by gel filtration on Sephadex G-50 in 0.01M-HCl (column size for 10mg of peptide: 1.0cm diam. × 50cm high, flow rate 20ml/h), freeze-drying, addition of triethylamine to a solution of the dried peptide in dimethyl sulphoxide until the point of precipitation and finally drying down. Fragment C has limited solubility in any solvent, particularly at higher pH; it is most soluble as the HCl salt.

Preparative coupling mixtures were gel-filtered on a small column (10cm diam. × 50cm high, flow rate 20ml/h) of Sephadex G-50 in 7% formic acid. The peaks eluted were collected, freeze-dried and examined spectrophotometrically (in order to quantify the extent of coupling), by the dansylation technique for end-group analysis, and by amino acid analysis (Table 6).

Test, and, later, preparative couplings were performed with 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (König & Geiger, 1970) instead of 1-hydroxybenzotriazole, in exactly the same manner as described above.

We also tried direct coupling, without pre-activation, by mixing 8μl of a solution containing 1μmol of fragment B_{-1}, in dimethyl sulphoxide, 1μl each of 1M solutions of dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole (or the other triazine mentioned above) and 10μl of a 100mM solution of the methyl ester of fragment $_{+1}C$ (prepared from fragment $_{+1}C$ in the same manner as methylated fragment B).

The direct-coupling method was found to be unsatisfactory: yields of coupled product were 3% (after 3h) and 7% (after 20h), relative to amino component.

We find, like Rees & Offord (1976*b*), that pre-activation of the carboxyl component is satisfactory. With 1-hydroxybenzotriazole as the esterifying agent coupling yields of 30–35% are the norm; 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine in a single quantitative trial gave a yield of 40%.

We therefore used pre-activation to prepare semisynthetic fragment BC and its analogues by the coupling of B_{-1} and $_{+1}C$ derivatives (Fig. 5). However, although satisfactory coupling between fragment B_{-2} and the test tetrapeptide was obtained, fragment B_{-2} would not couple to any of the $_{+2}C$ derivatives in Table 4. These latter materials also give poor colour yields with ninhydrin when stained on paper; it is possible that approach to the amino groups of $_{+2}C$ derivatives is sterically hindered.

Table 6. *Amino acid compositions and end groups of analogues of fragment BC*

The predicted values are based on the sequence (Margoliash *et al.*, 1961). Proline (expected value 2) was present but not determined. Where no value is given for any other amino acid, it was present in amounts less than the equivalent of a molar ratio of 0.1. n.d., Not determined.

Amino acid	Fragment BC (predicted)	Fragment BC (semisynthetic)	[F-Phe^{82}]-Fragment BC	des-Lys^{79}-[F-Phe^{82}]-fragment BC	des-Met^{80}-fragment BC
Aspartic acid	3	3.3	3.1	2.9	2.9
Threonine	3	2.9	3.1	2.9	2.5
Glutamic acid	5	4.9	5.0	4.9	4.8
Glycine	2	2.1	2.3	2.4	2.1
Alanine	3	3.3	2.7	3.0	3.2
Methionine	1	0.6	1.0	1.0	0.0
Isoleucine	4	2.8	3.1	3.0	3.0
Leucine	3	2.9	2.9	3.0	2.8
Tyrosine	3	2.3	3.4	2.1	2.4
Phenylalanine	1	0.9	—	—	1.1
Lysine	8	8.0	8.0	7.0	7.9
Arginine	1	0.9	0.8	1.1	1.2
N-Terminal group	Glu	Glu	n.d.	n.d.	Glu

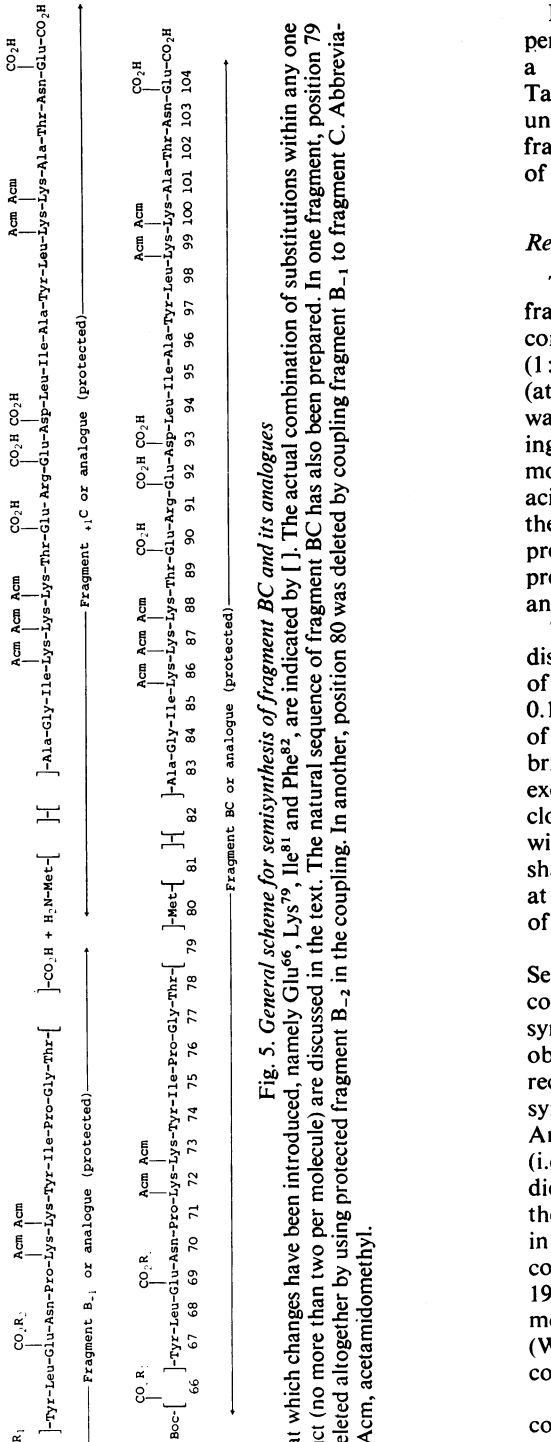

Fig. 5. *General scheme for semisynthesis of fragment BC and its analogues*

Sites at which changes have been introduced, namely Glu[66], Lys[79], Ile[81] and Phe[82], are indicated by [ ]. The actual combination of substitutions within any one product (no more than two per molecule) are discussed in the text. The natural sequence of fragment BC has also been prepared. In one fragment, position 79 was deleted altogether by using protected fragment B_{-2} in the coupling. In another, position 80 was deleted by coupling fragment B_{-1} to fragment C. Abbreviation: Acm, acetamidomethyl.

Fragment B_{-2} will couple to fragment $_{+1}C$ in a perfectly satisfactory manner, with the production of a semisynthetic des-Lys[79]-fragment BC (Fig. 5, Table 6). A preliminary experiment (C. J. A. Wallace, unpublished work) has produced [Asp[66],F-Phe[82]]-fragment BC by coupling the appropriate analogues of fragments B_{-1} and C_{+1}.

Re-formation of the complete protein

The methyl groups were removed from purified fragments BC by dissolving or suspending them, at a concentration of 0.5mg/ml, in triethylamine/water (1:49, v/v), which had been adjusted to pH10.5 (at 20°C) with solid CO_2. After 24h at 20°C, the pH was adjusted to 7 with 1M-acetic acid, and the resulting solution freeze-dried. The Boc group was removed by treatment in anhydrous trifluoroacetic acid at 4°C for 1h at a concentration of 1mg/ml. At the end of the treatment the product was recovered by precipitation with 4 vol. of cold diethyl ether. The precipitate was washed once with cold diethyl ether and dried.

To 0.25 μmol of such a product dissolved in 0.1ml of distilled water we added, cautiously, 0.5 μmol (4mg) of acetimidylated fragment A dissolved in 2ml of a 0.1M-potassium phosphate buffer, pH7.0. The vessel, of about 2.5ml capacity, was then topped up to the brim with the phosphate buffer. We then added an excess of $Na_2S_4O_7$ (1mg) and sealed the vessel with a closure pierced by a pin. The pinhole was then covered with Parafilm so as to exclude all air. The vessel was shaken to dissolve the solid and left for 24h in the dark at 20°C. These conditions are loosely based on those of Corradin & Harbury (1974).

We purified the product by gel filtration on Sephadex G-50 in 7% formic acid using the small column described above. When coupling with semisynthetic fragment BC of natural sequence we have obtained yields of up to 10%, comparable with the 6% recorded by Barstow *et al.* (1977) for their totally synthetic sample of fragment BC of natural sequence. Analogues in which the sequence changes are great (i.e. des-Lys[79]-fragment BC, des-Met[80]-fragment BC) did not couple. Such a result is not unexpected, since these analogues might well not fold about fragment A in such a way as to permit the sterically assisted coupling discovered by Corradin & Harbury (1970, 1974). These fragments will have to be coupled by the more conventional methods, which have been shown (Wallace, 1976, 1978) to be capable of re-forming the complete sequence with up to 30% efficiency.

In preliminary experiments, we have formed a non-covalent complex between acetimidyl-fragment A and semisynthetic acetimidyl-fragment BC in which the *N*-terminal residue, glutamic acid-66, appears to have cyclized to pyrrolidonecarboxylic acid (this

cyclization is frequently a cause of poor coupling yields between fragments A and BC). The complex is analogous to that between two tryptic fragments of the cytochrome (cytochrome *c*-T; Harris & Offord, 1977). The complex between CNBr fragments has about 50% of the biological activity of the intact protein in the depleted-mitochondria assay described above: this fact offers some promise that those semisynthetic analogues of fragment BC that will not undergo the Corradin & Harbury coupling could be used to give informative results in the non-covalent system. The covalent product, semisynthetic acetimidylated [Hse^{65}]cytochrome *c*, prepared by the above methods, has the expected amino acid analysis (Wallace, 1978). That it is in a satisfactory state from the point of view of tertiary, as well as primary, structure is indicated by the fact that visible spectroscopy shows it to be reducible by ascorbate and that it has a biological activity of between 84 and 87% of that of acetimidylated [Hse^{65}]cytochrome *c* of completely natural origin when assayed in the depleted-mitochondria system. The assays, which were carried out in the concentration range 2–6μM (53–87% of the maximal O_2 uptake), used material that had not been treated to remove denatured molecules.

Discussion

We draw the following conclusions.

(1) The acetimidyl group is suitable for amino-group protection in semisynthetic work on cytochrome *c*. Since biological activity is retained on protection, deprotection at the end of the semisynthesis is not necessary. Unlike more conventional forms of protection the group does not depress solubility. Carboxypeptidases A and B show a useful difference in specificity toward N^{ϵ}-acetimidyl-lysine.

(2) CNBr fragments are suitable intermediates for semisynthetic work on the protein, even when it is not possible, or desirable, to take advantage of the spontaneous aminolysis reported by Dyckes *et al.* (1974) and by Corradin & Harbury (1974). The ability to open and close the lactone ring of homoserine is most advantageous and the residue can readily be removed and replaced by methionine when required.

(3) The CNBr fragments can easily be modified by stepwise truncation followed by stepwise resynthesis. Residues removed from the *C*-terminus of a fragment destined to donate its α-carboxy group in a coupling are best replaced on to the *N*-terminus of the other partner in the coupling before the fragments are condensed.

(4) The methods developed by Rees & Offord (1976*b*) for the condensation of suitably protected tryptic fragments are equally applicable to CNBr fragments. The use of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine instead of 1-hydroxybenzotriazole gives comparable yields. So far, we have not found that direct coupling, without pre-formation of the active ester of the carboxyl component, is as successful.

(5) The semisynthetic fragment BC and its analogues are now ready for deprotection and coupling to fragment A (residues 1–65) to re-form the complete protein. The analogue [Hse^{65}]cytochrome *c*, prepared by these means, has satisfactory characteristics on chemical analysis and assay of biological activity.

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