Enzyme-assisted semisynthesis of polypeptide active esters and their use

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A method is described for the preparation of polypeptides activated uniquely at the C-terminus. The polypeptide is incubated in a concentrated solution of an amino acid active ester, the latter having its amino group free but adequately protected by protonation. The amino acid ester is coupled via its amino group to the C-terminus of the polypeptide by enzymic catalysis (reverse proteolysis). The resulting polypeptide C-terminal active ester is then isolated and coupled to a suitable amino component (generally a polypeptide) in a subsequent chemical coupling. The method appears to be generally applicable; fragments of horse heart cytochrome *c*, and porcine insulin, are used as examples. Two new analogues of cytochrome *c* have been prepared by using this method, with yields of up to 60% in the final coupling. Scope and limitations of the method are discussed.

INTRODUCTION

Great progress has been made in the field of protein semisynthesis [1] by using enzymes to form peptide bonds (for reviews see, e.g. [2,3]). Stepwise addition of amino residues (and of a few other small nucleophiles) to the C-terminus of polypeptide substrates may be achieved through use of the exopeptidase carboxypeptidase Y, or of endopeptidases such as trypsin. In addition to being capable of coupling a much wider range of small nucleophiles than is the case for carboxypeptidase Y (which is more susceptible to steric constraints), endopeptidases may, under some circumstances, be used to catalyse the condensation of two polypeptide fragments with great specificity and minimum use of protecting groups. However, two general problems are preventing more extensive development of enzyme-assisted semisynthesis and modification of proteins, namely those of condensation of large polypeptide fragments and of attachment to the C-terminus of reporter groups which are not good substrates for the chosen enzyme.

The first of these general problems arises because: (i) larger peptides are more likely to possess potential sites of undesirable cleavage by the endopeptidase used for the coupling; (ii) it is generally more difficult to obtain solutions of high molarity with larger peptides than with small peptides or small nucleophiles, so a favourable equilibrium is not easily attained; and (iii) larger peptides may present steric constraints (two large polypeptides and the enzyme have to come together) under the non-denaturing conditions of enzymic resynthesis (see, e.g., [4]). Since points (ii) and (iii) lead to long reaction times, this accentuates point (i).

The effects of all three of these points may be attenuated by using a high concentration of a small nucleophilic

agent (e.g. an amino acid active ester) in a rapid, preliminary, enzyme-assisted coupling to a carboxyl component. It is advantageous that only one of the long polypeptide sequences to be coupled needs to be exposed to the enzyme. Once isolated, the resulting modified polypeptide may be coupled to a suitable amino component in a chemical reaction. In this way, the second problem alluded to above, that of enzyme specificity, may be avoided, since the reporter group would be attached in a chemical, and not an enzymic, reaction.

In a preliminary communication [5], we pointed out that amino acid esters are, by virtue of their amino groups, suitable small nucleophiles to be attached to the C-terminus of a polypeptide chain in the initial enzymic coupling step. Of the amino acid esters that we tested, the 2,6-dichlorophenyl ester was particularly suitable and was found to be adequately protected against polymerization by the degree of protonation of the amino group under the conditions of enzymic coupling [5]. We have also reported [6] a technique involving enzymic attachment, to the C-terminus, of compounds which are the points for subsequent conjugate formation by reductive alkylation. In principle, both methods offer a means of coupling large fragments specifically through their C-termini, either to other such fragments or to small nucleophiles in dilute aqueous solution under non-denaturing conditions. Although the reductive alkylation method is useful for the specific attachment of reporter groups, it is not suitable for the construction of long unmodified polypeptide chains, since it connects C-terminus to C-terminus, and leaves a hydrophobic linker group in place.

The object of the present paper is to give fuller details of our active-ester approach and to illustrate it by

Abbreviations used: Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); the term 'acetimidylated' is used as in Proudfoot *et al.* [8], but strictly the product of the reaction of an amine with methyl acetimidate is a substituted acetamidine; Ala-OSu·HCl, L-alanine *N*-hydroxysuccinimide ester hydrochloride; Ala-ONp·HCl, alanine *p*-nitrophenyl ester hydrochloride; f.a.b.m.s., fast-atom-bombardment mass spectrometry.

presenting its successful application to the semisynthesis of a cytochrome *c* analogue. The method is more widely applicable, and its scope and limitations are discussed.

MATERIALS AND METHODS

Except where otherwise specified, reagents and solvents were obtained from commercial sources, were of analytical grade (or better) and were used without purification. Porcine trypsin was obtained from Sigma and was treated with Tos-Phe-CH₂Cl according to the established procedure [7]. Fully acetimidylated horse heart cytochrome *c* fragments containing residues 1–38 [acetimidyl-(1–38)-fragment] and 40–104 [acetimidyl-(40–104)-fragment, α -amino-group-free], were prepared by methods which have previously been described [8,9]; protected cytochrome fragment 40–104 was prepared from protected cytochrome fragment 39–104 by a single step of Edman degradation on the fragment 39–104 and was purified by ion-exchange chromatography [8,9]. The Ala-OSu·HCl and Ala-ONp·HCl were the products of Nova Biochem and Bachem respectively.

T.l.c. was performed on cellulose or silica sheets (Machery Nagel and Co. or Merck respectively) using either solvent system 1 (chloroform/methanol, 9:1, v/v) or system 2 (butan-1-ol/acetic acid/water/acetone, 7:2:4:7, by vol.), as specified. Spots were revealed with Cd/ninhydrin reagent; when necessary (that is, when acid-labile amino-protecting groups were present), sheets were exposed to the vapour of 12 M-HCl before staining.

H.p.l.c. was performed on a system that has previously been described [10], except that the U6K injector was replaced with a Wisp automatic injector (Waters). A C₁₈ 10 μ m Radialpak μ Bondapak cartridge (Waters) was used at a flow rate of 1 ml/min. Solvent A was prepared by adding 1 ml of trifluoroacetic acid (h.p.l.c. grade, Pierce) to 1 litre of h.p.l.c.-grade water (Milli Q system). Solvent B was pure acetonitrile (h.p.l.c. grade, Merck).

Mass spectra were obtained by using a Kratos MS 50 S machine operated under control of a DS 55 M data system (Kratos Ltd., Manchester, U.K.). The fast-atom-bombardment accessories were from M-Scan Ltd., Silwood Park, Sunninghill, Bucks., U.K.

Preparation of L-alanine 2,6-dichlorophenyl ester

To 3.78 g of *t*-butyloxycarbonyl-L-alanine dissolved in 40 ml of ethyl acetate containing 3.25 g (1 equiv.) of 2,6-dichlorophenol, was added 4.13 g (1 equiv.) of dicyclohexylcarbodi-imide dissolved in 40 ml of the same solvent. After 17 h at 22 °C, a white cake was obtained by filtration and rotary evaporation of the filtrate. Recrystallization from hexane to which a few drops of ethyl acetate had been added yielded 4.13 g of product, which ran as a single spot on silica t.l.c. (solvent system 1; *R_F* of product 0.72; *R_F* of *t*-butyloxycarbonylalanine standard 0.12). Amino protection was removed by dissolving 4.13 g of product in 25 ml of trifluoroacetic acid and incubating for 1 h at 22 °C. Alanine 2,6-dichlorophenyl ester was recovered by rotary evaporation (no heating). The white crystalline material was dried under high vacuum [yield 5.6 g; some trifluoroacetic acid is still present; it is essential to remove most of the excess trifluoroacetic acid after deprotection of the *t*-butyloxycarbonyl amino acid active ester; this is most easily achieved by adsorption of the ester from aqueous acid solution on to a C₁₈ cartridge, washing with 0.1 %

trifluoroacetic acid and elution with a mixture of 0.1 % trifluoroacetic acid and acetonitrile (1:5, v/v); up to about 200 mg of ester may be adsorbed at a time to a 1 g C₁₈ cartridge (Analytichem International, Harbor City, CA, U.S.A.) and characterized by fast-atom-bombardment mass spectrometry (f.a.b.m.s.); an intense protonated molecular-ion cluster was observed at *m/z* 234, 236, 238, as expected. The product ran as a single spot on t.l.c. (silica sheet; solvent system 2; *R_F* 0.61; *R_F* values for protected ester, *t*-butyloxycarbonylalanine and alanine standards under these conditions were > 0.9, 0.75 and 0.19 respectively). Analogous procedures, with slight modifications, were used to prepare and characterize three other esters of alanine after removal of butyloxycarbonyl protection: the 4-chlorophenyl ester was precipitated from trifluoroacetic acid solution by addition of nonane and cooling; the 2,4,5-trichlorophenyl ester and the 8-quinolinyl ester were rather unstable and were obtained as mixtures of the active ester and free alanine.

Tests of stability of amino acid active esters

The *N*-hydroxysuccinimide (OSu), 4-chlorophenyl and 2,6-dichlorophenyl esters of L-alanine (0.12–0.5 M) were incubated at 22 °C in aqueous butane-1,4-diol solution adjusted to pH values of between 4 and 6 (uncorrected glass electrode) with sodium acetate. Ala-ONp·HCl was studied in aqueous solution (0.12 M). Portions were withdrawn at intervals and analysed by t.l.c. (silica sheets, solvent system 2; cellulose sheets were used for Ala-ONp, since it decomposed on silica), by reversed-phase h.p.l.c., and, in the case of Ala-OSu, by f.a.b.m.s.

Enzyme-assisted semisynthesis of polypeptide active esters

A solution of butane-1,4-diol containing the active ester (0.2–0.5 M) was adjusted to a series of pH values (uncorrected glass electrode) with a saturated solution of anhydrous sodium acetate in butane-1,4-diol. The polypeptide substrate (acetimidyl fragment 1–38 of cytochrome *c*, 1.1 mM final concn.) was dissolved in the ester solution by the addition of about 10 % water by volume. It was found preferable to dissolve the polypeptide in the required amount of water and then to add the ester solution. Tos-Phe-CH₂Cl-treated porcine trypsin was added as a solution in water (25 mg/ml; enzyme/substrate ratio 1:4, w/w). During incubations at 22 °C, portions were removed, quenched with an equal volume of acetic acid and analysed by reversed-phase h.p.l.c.

Aminolysis of the polypeptide active ester

The efficiency of coupling of both small molecules and sizeable polypeptides to peptidyl active esters was tested in both organic and aqueous solvents. The conditions employed for aminolysis in dimethyl sulphoxide solution were described in a preliminary paper [5]. The conditions for aminolysis in aqueous solution are as follows; in all cases the polypeptidyl-active-ester concentration was 0.25 mM. The amino components, cytochrome *c*-(40–104)-fragment and -(39–65)-fragment and tyrosine ethyl ester, were 0.25 mM, 2.5 mM and 10 mM respectively. Carboxy and amino components were combined in distilled water so that, since the polypeptide active ester had been freeze-dried from acid solution, the resulting pH was low. A crystal of sodium dithionite was added to each mixture (generally 2 ml final volume), followed by

concentrated phosphate buffer of either pH 6 or 7 such as to obtain a phosphate concentration of 400 mM. Final adjustment of pH was made with 2 M-NaOH, and the mixture was kept sealed, at room temperature, in a glass-walled syringe for the required period. Incubation was terminated by gel filtration on Sephadex G-50 in 7% (v/v) formic acid, or, in the case of couplings to tyrosine ethyl ester, by cation-exchange chromatography on Trisacryl SP. Coupling yields were estimated from the relative peak heights recorded by monitoring the column effluent at 280 nm. Portions of product fractions were taken for amino acid analysis.

RESULTS AND DISCUSSION

Stability of amino acid active esters

Any decomposition of amino acid active ester to free amino acid or to dioxopiperazine affects an enzymic coupling reaction only to the extent that it reduces the concentration of desired nucleophile. Polymerization of the active ester, on the other hand, is undesirable, since the resulting oligomers would still have a free amino group and so might be coupled by the enzyme to the C-terminus of the polypeptide fragment; however, this problem did not seem to occur in the experiments described here. This is probably because, in view of the high relative concentration of amino acid ester over decomposition products, formation of dipeptide ester is the most likely route for the decomposition of the starting material, and such a compound rapidly decomposes further to a dioxopiperazine.

Acylation of butane-1,4-diol by the active ester would also be undesirable, since the resulting ester would be expected to couple enzymically, but this phenomenon was not observed with the chlorophenyl or dichlorophenyl esters under the conditions described here.

Once the amino groups were deprotected, the 8-quinoliny ester, and particularly the trichlorophenyl ester, could not be obtained free from alanine and were found to decompose very rapidly in aqueous solution; the 8-quinoliny ester was hydrolysed completely within 30 min at pH 4. Ala-OSu was found to be too unstable to be useful for enzyme-assisted synthesis; f.a.b.m.s. showed that Ala-OSu rapidly acylated the solvent, butane-1,4-diol, even at low pH. Decomposition of the chlorophenyl and dichlorophenyl esters was less than about 10% (visual inspection of t.l.c. sheets) over a period of 2 h at pH values of 6 or below and appeared limited to simple hydrolysis. These last two esters, and the commercially-available nitrophenyl ester, were selected for enzymic coupling studies.

Enzyme-assisted coupling of amino acid active esters

Table 1 shows the results of the optimization of the coupling of alanine dichlorophenyl ester to acetimidyl cytochrome *c*-(1-38)-fragment at pH 5.5, a pH chosen to be at the high end of the range of ester stability. The reaction is under thermodynamic control. Since the peptide active ester produced is quite stable at this pH, the yield tends to reach a plateau. The coupling of alanine monochlorophenyl ester and Ala-ONp to acetimidyl-(1-38)-fragment produced similar results (Table 1), with yields, also at pH 5.5, of 60% after 120 min and 40% after 20 min respectively. The polypeptidyl *p*-nitrophenyl ester was found to be rather unstable once formed, even when the reaction mixture was quenched with formic acid. With des-Ala^{B30}-insulin as substrate

Fig. 1. Isolation of acetimidyl-cytochrome *c*-(1-38)-fragment-alanine dichlorophenyl ester by ion-exchange chromatography

The exact conditions of the enzymic attachment of alanine 2,6-dichlorophenyl ester to acetimidyl-cytochrome *c*-(1-38)-fragment, and of the ion-exchange separation were as previously described [5]. Peaks: SR, solvent and reagents; T, trypsin; A, uncoupled acetimidyl-(1-38)-fragment position; B, product identified as acetimidyl-cytochrome *c*-(1-38)-fragment-alanine dichlorophenyl ester, which was pooled as shown. The separation was improved by using a shallower gradient (results not shown). The pooled product peak was desalted by gel filtration on a column (30 cm × 2.2 cm diam.) of Sephadex G-25 eluted with 1% (v/v) acetic acid, and was recovered by freeze-drying.

Table 1. Yields of polypeptide active esters

The Table shows the yields, determined by h.p.l.c., of polypeptide active ester obtained when attaching various esters of alanine to the polypeptide substrate acetimidyl-cytochrome *c*-(1-38)-fragment at pH 5.5, under conditions described in the Materials and methods section. Concentrations of amino acid esters were 0.24, 0.43 and 0.19 M for the monochlorophenyl, dichlorophenyl and 4-nitrophenyl ester respectively.

Ester	Time (min) . . .	Yield (%)					
		20	30	60	120	180	240
4-Chlorophenyl	—	—	52	—	—	60	—
2,6-Dichlorophenyl	—	—	21	37	51	57	61
4-Nitrophenyl	40	—	—	29	—	—	—

and alanine dichlorophenyl ester as nucleophile, studied at pH 5.5, a yield of 75% was obtained after 180 min.

Preparative isolation of polypeptidyl active esters

The product of the coupling of alanine dichlorophenyl ester to the C-terminus of acetimidyl-cytochrome *c*-(1-38)-fragment was isolated by ion-exchange chromatography (Fig. 1). From the absorbance profile of the isolation, and on the basis of equal absorbance at 280 nm of coupled and uncoupled product (verified by spectrophotometry of purified material), the coupling yield is approx. 61%. A similar isolation involving alanine monochlorophenyl ester led to an estimated yield of coupled product of 75%. The apparent yield of polypeptide monochlorophenyl ester was thus higher than that found by h.p.l.c. in the original, small-scale, optimization experiments. In the case of alanine dichlorophenyl ester, polypeptide active ester was recovered after ion-exchange chromatography, gel filtration and freeze-drying in a yield about 40% on the basis of acetimidyl-cytochrome *c*-(1-38)-fragment. Porcine insulin dichlorophenyl ester^{B30} the product of the coupling of alanine dichlorophenyl ester to des-Ala^{B30}-insulin was isolated in about 50% yield under similar conditions.

Characterization

Acetimidyl-cytochrome *c*-(1-38)-fragment-alanine dichlorophenyl ester, isolated by ion-exchange chromatography followed by gel filtration (Fig. 1), was analysed by reversed-phase h.p.l.c. (Fig. 2) and found to be contaminated with only a small amount of uncoupled or hydrolysed material. Similar results were obtained with

Fig. 2. H.p.l.c. chromatogram of polypeptide active ester

The Figure shows an analytical trace of acetimidyl-cytochrome *c*-(1-38)-fragment-alanine dichlorophenyl ester isolated as fraction B (see Fig. 1). Conditions were as described in the Materials and methods section. The column was equilibrated with 100% solvent A, eluted isocratically for 5 min, then a linear gradient of solvent B was applied (1%/min) to 55% B. A small fraction of the material is seen to be hydrolysed and is eluted at the position of acetimidyl-cytochrome *c*-(1-38)-fragment (arrow 1). The major peak (arrow 2) was characterized as acetimidyl-cytochrome *c*-(1-38)-fragment-alanine dichlorophenyl ester. 'Inj' indicates where the sample was injected.

acetimidyl-cytochrome *c*-(1-38)-fragment-alanine monochlorophenyl ester, which was eluted on h.p.l.c. a little earlier than the dichlorophenyl ester, and with insulin dichlorophenyl ester^{B30}. The acetimidyl-(1-38)-fragment active esters were digested by porcine trypsin in phosphate buffer at pH 6 at room temperature. This released the expected ester of alanine, which was identified by t.l.c., and the original polypeptide, which was identified by h.p.l.c. (results not shown). As expected, the alanine esters did not decompose at the pH at which the digestion was performed.

Aminolysis in an organic solvent

In order to study the aminolysis reaction, acetimidyl-cytochrome *c*-(1-38)-fragment-alanine dichlorophenyl ester and the corresponding monochlorophenyl ester were separately mixed, in dimethyl sulphoxide, with threonine t-butyl ether t-butyl ester as nucleophile (about 15 mM-polypeptidyl ester, 1 M-nucleophile, 22 °C). Analytical h.p.l.c. showed (Fig. 3), after 2 h in the case of the dichlorophenyl ester, disappearance of the polypeptidyl active ester and the production of a new peak at a longer retention time. This later peak, together with the peak corresponding to ester hydrolysis, were isolated by preparative h.p.l.c. and characterized [5] by tryptic digestion (Fig. 4 for the dichlorophenyl ester). Aminolysis in the case of the monochlorophenyl ester was still not complete even after 24 h under these conditions, and the yield of product was only about 35%.

From the results of the above experiments we concluded that the dichlorophenyl ester possessed the

Fig. 3. H.p.l.c. chromatogram of aminolysed polypeptide active ester

The Figure shows an analytical h.p.l.c. trace of the aminolysis reaction mixture containing acetimidyl-cytochrome *c*-(1-38)-fragment-alanine dichlorophenyl ester (~15 mM) and threonine t-butyl ether t-butyl ester (1 M) in dimethyl sulphoxide solution, after 2 h at 22 °C. Elution conditions were as for Fig. 2. Virtually no active ester (position of arrow 2) remains. The most prominent peak (arrow 3) is due to expected product, acetimidyl-cytochrome *c*-(1-38)-fragment-alanylthreonine t-butyl ether. Some hydrolysis of the dichlorophenyl ester occurred to give acetimidyl-cytochrome *c*-(1-38)-fragment-alanine, which is eluted under these conditions at the position of acetimidyl-cytochrome *c*-(1-38)-fragment (arrow 1). Peaks 1 and 3 were isolated by preparative h.p.l.c. and characterized by tryptic digestion. 'Inj' indicates where the sample was injected.

Fig. 4. H.p.l.c. chromatogram of trypsin-digested acetimidyl-cytochrome *c*-(1-38)-fragment-alanylthreonine *t*-butyl ether *t*-butyl ester

The Figure shows an h.p.l.c. trace of the product of trypsin digestion of acetimidyl-cytochrome *c*-(1-38)-fragment-alanylthreonine *t*-butyl ether *t*-butyl ester (peak 3 of Fig. 3). Elution conditions were as for Fig. 2. Complete digestion was achieved, and the digested protein product ran at the position of acetimidyl-cytochrome *c*-(1-38)-fragment (arrow 1), as expected. The dipeptide derivative, alanyl-*t*-butylthreonine *t*-butyl ester, was isolated from the digest and identified by f.a.b.m.s. (results not shown). 'Inj' indicates where the sample was injected.

most useful combination of stability and reactivity amongst the esters tested. An attempted aminolysis [5] of acetimidyl-cytochrome *c*-(1-38)-fragment-alanine dichlorophenyl ester with acetimidyl-cytochrome *c*-(40-104)-fragment was not very successful, most probably because the peptide amino component (about 11 mM, close to the limit of solubility) was not able to compete effectively with hydrolysis of the active ester by traces of moisture under the harsh conditions employed (presence of excess di-isopropylethylamine, a relatively strong base).

When attempting to condense two large polypeptides, it is not possible to maintain a concentration of the order of 1 M apparently necessary for aminolysis to compete effectively with hydrolysis, even when reasonable care is taken to keep the dimethyl sulphoxide dry. Although some improvement of the aminolysis-to-hydrolysis ratio might be expected through even more rigorous drying of polypeptide reagents and glassware, and through use of a milder organic base such as *N*-methylmorpholine, this is not a very convenient approach and may lead to irreversible denaturation of some polypeptides.

Aminolysis in aqueous solution

Another way to permit aminolysis to compete with hydrolysis is, paradoxically, to perform the reaction in water, but at a much lower effective pH than was used for the couplings in dimethyl sulphoxide. α -Amino groups are then largely deprotonated, but the hydroxide-ion concentration is low. Reaction may be assisted, where necessary, by the high solubility of peptides in water, and, in certain cases, by a specific association that may obtain between large peptides derived from limited

Fig. 5. Comparison of the coupling efficiency of activated acetimidyl-cytochrome *c*-(1-38)-fragment under a variety of conditions

Experimental conditions were as indicated in the Materials and methods section. $\circ$, Coupling of acetimidyl-[Ala³⁹]-cytochrome *c*-(1-39)-fragment 2,6-dichlorophenyl ester (lot I) and acetimidyl-cytochrome *c*-(40-104)-fragment, for 24 h; $\triangle$, the same for 1 h; $\blacktriangle$, the same for 10 min; $\square$, the same (lot II, of the polypeptide active ester), 1 h; $\blacksquare$, acetimidyl-[Ala³⁹]cytochrome *c*-(1-39)-fragment 4-chlorophenyl ester and acetimidyl-cytochrome *c*-(40-104)-fragment, 24 h; $\times$, acetimidyl-[Ala³⁹]cytochrome *c*-(1-39)-fragment 2,6-dichlorophenyl ester (lot I) + acetimidyl-cytochrome *c*-(39-65)-fragment; 24 h; $+$, acetimidyl-[Ala³⁹]cytochrome *c*-(1-39)-fragment 2,6-dichlorophenyl ester (lot I) and tyrosine ethyl ester, 1 h.

proteolytic cleavage (see, e.g., [8,11]). The complex of cytochrome-*c*-(1-39)-fragment and -(40-104)-fragment is an example of such a special case [9], so it was conceivable that, by having an activated form of acetimidyl-(1-39)-fragment, particularly high coupling yields might result.

Fig. 5 summarizes the results obtained with the dichlorophenyl ester of acetimidyl-cytochrome *c*-(1-38)-fragment-alanine dichlorophenyl ester under a variety of conditions. The pH profile shows an optimal reactivity at pH 8.5. α -Amino groups in proteins and polypeptides generally exhibit pK_a values of about 7.5-8 [12], so that the amino group of acetimidyl-cytochrome *c*-(40-104)-fragment would be expected to be largely deprotonated. The time course for the reaction shows it to be complete at 60 min, and half complete at 10 min. In the case of one preparation of the polypeptidyl active ester the optimal yield was 30%, and for another it was 45%. This difference may reflect different degrees of spontaneous hydrolysis on work-up and storage. The product peak was shown in both cases, by amino acid analysis, to be 18-*N*-acetimidyl-[Ala³⁹]cytochrome *c*. We determined the amino acid composition of this analogue as the mean of three determinations (except for lysine and arginine, the values for which were lost for one of the three analyses). The result was as follows (expected values in parentheses; italic type for the two values that change with respect to the native cytochrome; the 'Lys'

value is the sum of acetimidyl-lysine and the ~ 15 % free lysine formed during acid hydrolysis): Asp, 8.6 (8); Thr, 9.3 (10); Glu, 12.9 (12); Gly, 12.1 (12); Ala, 7.0 (7); Val, 3.3 (3); Met, 1.4 (2); Ile, 4.9 (6); Leu, 5.9 (6); Tyr, 3.2 (4); Phe, 3.8 (4); His, 3.5 (3); Lys, 18.0 (18); Arg, 2.3 (2). A second analogue, des-(40-55)-[Ala³⁹]cytochrome *c*, was prepared by similar methods with a coupling yield of 60 % [13].

The monochlorophenyl ester was also tested under the optimum conditions determined by the experiments with the dichlorophenyl ester, but gave only 10 % yield, confirming our experience with small-molecule amino components in dimethyl sulphoxide.

When we employed, as amino components, cytochrome *c*-(39-65)-fragment and tyrosine ethyl ester under optimal conditions, yields were also lower than obtained with (40-104)-fragment (see Fig. 5). In contrast with (40-104)-fragment, (39-65)-fragment does not complex with (1-39)-fragment, as judged by gel filtration, and we concluded that the elevated yields obtained with the (1-39)(40-104) system can probably be attributed to conformational assistance. Nonetheless, the coupling yields obtained for the condensation of non-complexing fragments are quite satisfactory, especially since, under the conditions employed, the reaction is both rapid and simple to perform. The medium does not cause product denaturation, nor does it lead to side reactions. Furthermore, and because of the above factors, both carboxy and amino components can be recycled.

Conclusion

We have demonstrated that it is possible to prepare, in reasonably good yield and without the need for ω -carboxyl protection, polypeptides carrying a single activating group specifically placed by enzymic means at the C-terminus, and that it is possible to exploit such activation to couple, in a subsequent chemical reaction, a small nucleophile or a large polypeptide. We have prepared two different analogues of cytochrome *c* in yields of up to 60 %, a value that is difficult to obtain when coupling together large polypeptide fragments by other methods. Because of the facility and specificity of both activation and coupling steps, the method will be a valuable addition to the techniques of fragment condensation semisynthesis, particularly since it will be possible to use enzymes other than trypsin for the synthesis of polypeptide active esters. We chose to work with porcine trypsin as catalyst because of its ability to function under conditions of high concentration of organic solvents and relatively low pH [14]. Preliminary data show that the chymotryptic cytochrome *c*-(1-36)-fragment can be activated in a similar manner, and that esters of amino acids other than alanine may be used (C. J. A. Wallace, K. Rose & A. E. I. Proudfoot, unpublished work). In view of the size of the preferred active ester group, 2,6-dichlorophenyl, and the possibility of enzyme-catalysed oligomerization, it is doubtful whether the exopeptidase carboxypeptidase Y would be useful.

The work described here mostly concerns amino-protected fragments of cytochrome *c*. However, the amino protection is not really necessary during the enzymic coupling of the amino acid dichlorophenyl ester,

since, under the optimum conditions described above, the amino groups are adequately protected by protonation. Thus we have successfully prepared an activated derivative of an unprotected fragment of cytochrome *c* [cytochrome *c*-(1-38)-fragment-alanine dichlorophenyl ester; A. E. I. Proudfoot & C. J. A. Wallace, unpublished work] and a specifically activated derivative of unprotected insulin (trypsin was used to form a peptide bond between the carboxy group of Lys^{B29} and the amino group of alanine dichlorophenyl ester; K. Rose, unpublished work). It is only when the resulting polypeptide ester is used in the less acidic conditions required for coupling that protection is normally required, so as to prevent reaction through the wrong amino groups. However, even here, we expect that protection can be avoided if the coupling of the two fragments is assisted by the intermediate formation of a non-covalent complex (cf. [15]).

We believe that we have established that our method is likely to be a general one, and we expect that the techniques described here will be useful to those engaged in the semisynthesis and chemical modification of a wide variety of proteins.

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