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The Possible Use of Cyanogen Bromide Fragments in the Semisynthesis of Proteins and Polypeptides

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Peptide semisynthesis

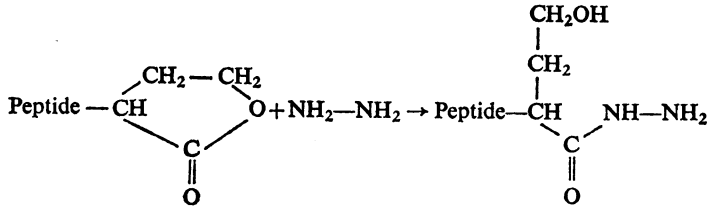
The chemical synthesis of proteins and peptides is accomplished by coupling together suitably protected amino acids or peptides. Since the desired products are almost invariably closely similar to a natural sequence, it is worth while to attempt a semisynthetic route: in other words to attempt to use naturally obtained fragments of proteins and peptides as ready-made intermediates in the synthesis. The technical problems of reversible protection and coupling that are involved have now largely been overcome both for the semisynthetic analogue of the stepwise-condensation method (Borras & Offord, 1970) and for the semisynthetic analogue of fragment condensation (Backer & Offord, 1968; Offord, 1969*a*; Hayward & Offord, 1971; W. H. Johnson, R. E. Offord & A. R. Rees, unpublished work).

Cyanogen bromide peptides

The fragments so far prepared for condensation have been tryptic peptides, varying in length from 5 to 39 residues. The present paper suggests a possible means of using fragments cleaved from proteins by the attack of CNBr (Gross & Witkop, 1962) on methionine residues. Such fragments have two particular advantages that render their use complementary to that of tryptic peptides. The first is that methionine residues occur relatively infrequently and so, if it were to be possible to use them, one would be coupling fewer, larger, fragments. The second advantage is that, if the substitution of homoserine for methionine is desired for the final product, or can be tolerated in it, the fragments cleaved from a protein can be used for coupling with very little extra treatment. All that is required is that the amino groups should be reversibly protected. This is because when CNBr cleaves the chain it converts the methionine into homoserine and leaves it as the *C*-terminal residue of the newly liberated fragment in the form of the lactone. Thus the terminal carboxyl group is in the form of a (cyclic) ester, and it is the only one in the fragment to be in such a form. This means that the principal condition for semisynthesis by fragment condensation has been met, namely that a chemical distinction must be achieved between the α-carboxyl group and all others in the fragment.

This distinction may be exploited in two ways. In both of these the newly liberated α-amino group of the fragment must first be protected (see below), the other (ε) amino groups having normally been protected before the cleavage.

In the first type of route the amino-protected fragment is treated with hydrazine. Conditions (see below) have been found in which the sole action of the hydrazine is to open the cyclic ester, causing the fragment now to end with homoseryl hydrazide:



This fragment may be used directly for coupling by the well-established azide method. The usual method is to treat the hydrazide with acid t-butyl nitrite (Honzl & Rudinger, 1961). Alternatively, if the azide coupling is considered undesirable, the second type of route may be used, in which couplings are mediated by carbodi-imide (Sheehan & Hess, 1955, 1956). Here the side-chain carboxyl groups are reversible protected by esterification (Offord, 1969*a*; Hayward & Offord, 1971) while the homoserine is still in the lactone form. As a consequence, if the lactone is subsequently opened by mild basic treatment, the resulting terminal carboxyl group will be the only one free for coupling.

Preparing for an azide coupling

The peptide Lys-Gln-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-homoserine was obtained from bovine ribonuclease (grade 1-A; Sigma Chemical Co., St. Louis, Mo., U.S.A.) by CNBr cleavage and gel

filtration in 0.1M-acetic acid exactly as described by Gross & Witkop (1962). Approx. 85mg is obtained from 1g of protein, a yield of about 85%. Electrophoresis at pH6.5 showed that most of the peptide had a mobility m of +0.79 (relative to that of aspartic acid = −1, measured and interpreted as described by Offord, 1966), while a trace had a mobility of +0.58. These mobilities correspond to net charges of +4 and +3 respectively. This was interpreted to mean that peptide was still substantially in the lactonized form with just a trace of the open form.

Properties of the lactone ring. As a preliminary to the hydrazinolysis, a study was made of the opening and closing of the lactone ring in this peptide. An aqueous solution of triethylamine (2%, w/v) was adjusted to pH10 with solid CO_2 and used to bring about the opening of the ring. The time-course of the reaction was observed by taking out 5μl samples of a 2mM solution of the peptide in the triethylamine–carbonate reagent, freeze-drying them in a matter of seconds and, when the full series had been collected, running them at pH6.5; the expected change from $m = +0.79$ to $m = +0.58$ was found to be largely complete after 20–30min at 20°C. Longer times (up to 2h) were found to give progressively more of a band that was even less basic ($m = +0.37$, net charge = +2), which is attributed to loss of a labile amide group. The time-course of the ring closure was studied by a similar type of experiment in which a sample of the peptide that had been fully converted into the open form by using the optimum conditions established above was dissolved in anhydrous trifluoroacetic acid to a concentration of 3mM. The ring closure ($m = +0.58$ going to $m = +0.79$) appeared to be complete after 1h at 20°C.

Hydrazinolysis. Conditions for hydrazinolysis were established in a similar way. An $AgNO_3$–NH_3 spray for aminoacyl-hydrazides (Offord, 1969*b*) was found to be unsuccessful for hydrazides of the longer peptides, and the $FeCl_3$–$K_3Fe(CN)_6$ spray (Barton *et al.*, 1952), though sensitive, is not specific for hydrazides. Measurements of m at pH6.5 are less satisfactory as an indication for success in hydrazinolysis than they were for ring opening and closing, since the mobility of the peptide in the hydrazide form should, on the basis of the expected pK values of the –NH–NH_2 group, be similar to that of the lactone. It was therefore decided to seek to differentiate between these two forms of peptide, lactone and hydrazide, by the fact that the hydrazide would be stable to the basic conditions that were described above as suitable for opening the lactone. A time-course was accordingly studied, by means similar to those used for the preceding parts of this work, for the reaction between the peptide and hydrazine. The peptide was pretreated with trifluoroacetic acid to maximize the amount of the lactone form and washed with methanol. It was then dissolved (4mM) in a solution (10%, v/v) of anhydrous hydrazine in dimethylformamide. It was found that, after 6–10min of this treatment at 20°C, all the peptide had changed to a form in which m was unaffected by treatment with base. In fact m did change slightly as a result of hydrazine treatment (from +0.79 to +0.75) and the $FeCl_3$–$K_3Fe(CN)_6$ stain revealed an intense blue band with this mobility (the traces of hydrazine remaining after freeze-drying move with $m = +2.8$). After electrophoresis at the same loading (0.05μmol/cm), untreated peptide gave a very pale blue colour with the stain. It was therefore concluded that the hydrazinolysis was successful under these conditions and that deamidation had not occurred. Final conformation had to await the use of such materials for coupling.

Amino protection. The other essential step for the preparation of the fragment for coupling is amino protection. In the present case the fragment would only be used as a carboxyl component in a coupling since it represents the *N*-terminal section of the parent protein. Thus, exceptionally, it can be protected after cleavage and the same protecting groups can be applied to both the ϵ-amino groups and the α-amino groups. (However, in all cases, amino protection must be carried out before hydrazinolysis, or the –NH–NH_2 function will be protected as well.) Two alternative groups were used in the present study. The benzyloxycarbonyl group (Bergmann & Zervas, 1932) was applied as described by Offord (1969*a*) and the maleyl group by minor modifications of the method of Butler *et al.* (1969). The benzyloxycarbonyl-peptide does not migrate on electrophoresis at pH6.5 and is in fact insoluble under these conditions. The maleyl-peptide migrates with $m = -0.61$ for the lactone form and $m = -0.41$ for the open form. These values correspond to net charges of $-3\frac{1}{2}$ and $-2\frac{1}{2}$. The fit is thus not so good as is the case with all the other forms of this peptide, but could be explained by proposing that the histidine residue is less than fully charged in the maleyl derivatives. The amino-protected peptides are ninhydrin-negative, but may be located by specific sprays for arginine and histidine.

Preparing for a carbodi-imide coupling

The benzyloxycarbonyl-protected peptide was prepared and treated with anhydrous trifluoroacetic acid, as above, to return any homoserine that had opened to the lactone form. The peptide was suspended in ethanol–water (17:3, v/v) and the side-chain carboxyl groups were benzylated with phenyldiazomethane at a (glass electrode) pH of 4.5 as described by Offord (1969*a*). In the course of this reaction the peptide went completely into solution. The lactone was then opened with a 20min treatment with the triethylamine–carbonate reagent. That protection and ring opening had gone as expected was indicated by the results of parallel experiments on

peptide with free amino groups on which mobilities may be followed more easily. [For serious coupling work it is essential to protect the amino groups before esterification; see, e.g., Hayward & Offord (1971).] A sample of the unprotected peptide (the lactone form) gave on esterification a band of $m = +0.88$ (trailed) corresponding to a net charge of +5. Treatment with triethylamine–carbonate changed the mobility to +0.80 (trailed), the expected value. This last result also suggests that the benzyl ester groups are not saponified by the triethylamine. Once again, the final proof of the correctness of the protection of the amino and carboxyl groups must lie in successful coupling.

Peptide coupling

Preliminary experiments have been carried out in the coupling of 10mg samples of the appropriate peptide derivative to tyrosine ethyl ester. Yields were poor (about 10%) by both azide and carbodi-imide routes. The conditions for the azide route were those of Visser *et al.* (1970) and the conditions for the carbodi-imide route were those of Köning & Geiger (1970), with 1.2 equiv. of 1-hydroxybenzotriazole. Experiments carried out by C. Wallace (unpublished work) with benzyloxycarbonyl-homoserine as a model component indicate that the principal factor responsible for the low yields is that the carboxyl component tends to lactonize rather than couple. Yields should be improved by increasing the concentration of reactants. The model experiments indicate that the rearrangement to the peptide isocyanate (Curtius, 1904), a phenomenon that has been noted in azide coupling with serine (Fruton, 1942), does not occur to an appreciable extent. The products of both model and peptide couplings were fully digestible with proteases, and racemization is therefore not thought to constitute a problem. Even quite low coupling yields are acceptable if one considers that there are cases in which only one or two couplings are needed to introduce some useful synthetic modifications into proteins of considerable size. If the peptide lactone is the principal abortive product, the possibility also exists of recycling it through the appropriate part of the whole procedure.

The other drawback of the proposed method is that some methionine residues may be essential for activity and, if so, homoserine will often be unacceptable as a substitute. If the carbodi-imide route is to be used the homoserine could be removed enzymically with carboxypeptidase A. In favourable cases this could be done without any extensive further degradation of the fragment. Methionine would then be best attached to the *N*-terminus of the succeeding fragment.

In conclusion, it is proposed that, with all its obvious limitations, this approach should find a place in protein semisynthesis. Of small proteins cytochrome *c* seems the most appropriate for an extended trial, and of the larger ones triose phosphate isomerase from chicken muscle, with two methionine residues in a total of 250 (A. J. Furth, J. D. Priddle, J. D. Milman & R. E. Offord, unpublished work), seems suitable.

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