DETERMINATION OF THE AVAILABILITY OF CARBODIIMIDE-ACTIVATED N-PROTECTED AMINO ACIDS IN SOLID PHASE PEPTIDE SYNTHESIS

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SUMMARY

A new method is described for the determination of the availability of carbodiimide-activated N-protected amino acids in solid phase peptide synthesis. The method involves the addition of a second nucleophile to a solid phase coupling reaction at different time intervals and measuring the amount of activated amino acid intercepted. Using the DCCI-mediated coupling reaction of Boc-Ala-OH and H-Gly-O-resin with H-Gly-O-tBu as the second nucleophile, it was determined that ca. 61% of the theoretical maximum amount of activated Boc-Ala-OH was available after 8 hr of reaction.

Nearly since the introduction of solid phase peptide synthesis (SPPS) (1), much interest has been focused on how long should carbodiimide-mediated coupling reactions be run. Some investigators (2) felt that reaction times of 2 hr were sufficient because activated N-protected amino acid species would be exhausted after this period (3). This contention remained somewhat of a controversy as different points of view on the subject were based on personal experiences in SPPS.

In 1973, Tometsko (4) reported the results of his study deliberately undertaken to determine the availability of carbodiimide-activated N-protected amino acids in SPPS as a function of time. Tometsko employed a model system in which a solution of t-butyloxycarbonyl-L-leucine (Boc-

Leu-OH) (1.07 mmoles) dicyclohexylcarbodiimide (DCCI) (1.47 mmoles) and triethylamine (0.2 ml ~ 1.45 mmoles) in 50 ml of methylene chloride was allowed to preincubate for varying time periods (0-8 hr) before aliquots were removed and allowed to react with L-Alanine resin ester (H-Ala-O-resin). Formation of Boc-Leu-Ala-O-resin diminished linearly with preincubation time reflecting corresponding inactivation of the Boc-Leu-OH with preincubation time.

We, too, have been interested in determining quantitatively the availability of activated N-protected amino acids in carbodiimide-mediated couplings in SPPS as a function of time. We wish to report here another method for determining the availability of DCCI-activated N-protected amino acids which we believe is more meaningful than the Tometsko method, since it more closely simulates actual solid phase conditions throughout the determination.

MATERIALS AND METHODS

The initial phase of the procedure to determine the availability of activated N-protected amino acids in carbodiimide-mediated SSPS was carried out in the usual manner. A 0.75 g-portion of t-butyloxycarbonylglycine resin ester (Boc-Gly-O-resin) (5) corresponding to 0.41 mmoles of Gly was placed in a cylindrical solid phase reactor (6) that was equipped for agitation by nitrogen percolation during reaction and washings. The following sequence of steps were followed, (a) 30 min deblock of the Boc protective group with 1 N HCl in AcOH; (b) wash with AcOH (3X); (c) wash with EtOH (3X); (d) wash with DMF (3X); (e) 8-10 min treatment with 1 ml of triethylamine in 15 ml of DMF; (f) wash with DMF (3X) (the NEt_3/DMF filtrate and two of the DMF washing filtrates were collected, acidified, while cooling, with excess 5 N nitric acid and titrated potentiometrically with 0.1 N $AgNO_3$ to assure complete Boc deblocking); (g) wash with CH_2Cl_2 (1X); (h) add a solution of 0.189 g (1 mmole) of Boc-Alanine in 2-3 ml of CH_2Cl_2 and mix for 15 min; and (i) add a solution of 0.206 g (1 mmole) of DCCI in 2.5 ml of CH_2Cl_2 and the reaction mixture was agitated gently by N_2 percolation.

At time intervals of 2, 3, 5.3 and 8.5 hr (separate experiments for each time interval) after the addition of DCCI, a solution of 0.47 mmole of glycine t-butyl ester in 1 ml of CH_2Cl_2 was added and the reaction was allowed to continue for at least 3 hr. The reaction mixtures were filtered and washed with CH_2Cl_2, filtrate and washes were combined and retained. A check of the coupling efficiency, i.e., Boc-Ala-Gly-O-resin formation, after the 2 hr coupling time (1st experiment) by the pyridine hydrochloride method (7), showed that the resin coupling reaction was at least 97.5% complete. Combined filtrate and washings of each experiment were evaporated to dryness and the residue (Boc-Ala-Gly-O-tBu, H-Gly-O-tBu, Boc-Ala-dicyclohexylurea and dicyclohexylurea (DCU) was treated with trifluoroacetic acid for 40 min. After evaporation of trifluoroacetic acid, the residue was further dried in vacuo over KOH. The residue was dissolved in several milliliters of water and this solution was carefully decanted from an insoluble film of material, presumably DCU and N-acylurea. The evaporation flask was rinsed with another milliliter of water and the combined portions of the solution were neutralized with pyridine and made up to 5.0 ml. These solutions were then analyzed by chromatographing on a Phoenix automatic amino acid analyzer (8) that was calibrated for H-Ala-Gly-OH. The results are shown in Figure 1.

As check on the formation Boc-Ala-Gly-O-resin, the peptide product of the first experiment was decoupled from its resin support with HBr in trifluoroacetic acid during 90 min. After evaporation of acids, the peptide hydrobromide salt was precipitated with ether and there was obtained 89 mg (98%). Chromatography of the peptide salt on the analyzer showed it to be homogeneous.

RESULTS AND DISCUSSION

This method of determining the availability of activated N-protected amino acids in carbodiimide-mediated solid phase peptide synthesis is outlined in Scheme I. In essence, a solid phase coupling reaction is initiated

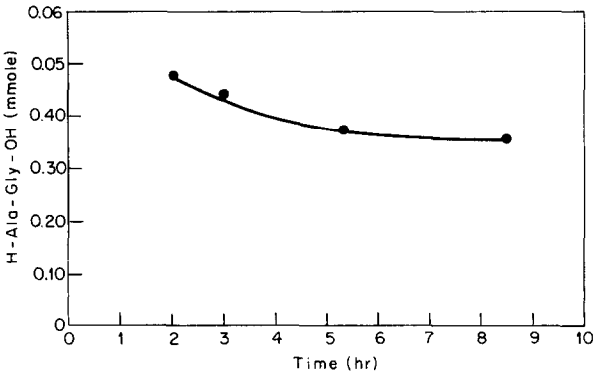

Figure 1. Formation of H-Ala-Gly-OH (after deblocking) after addition of H-Gly-O-t Bu (0.47 mmole) to dicyclohexl-carbodiimide (1 mmole)-mediated coupling of Boc-Ala-OH (1 mmole) and H-Gly-O-resin (0.41 mmole) after time intervals.

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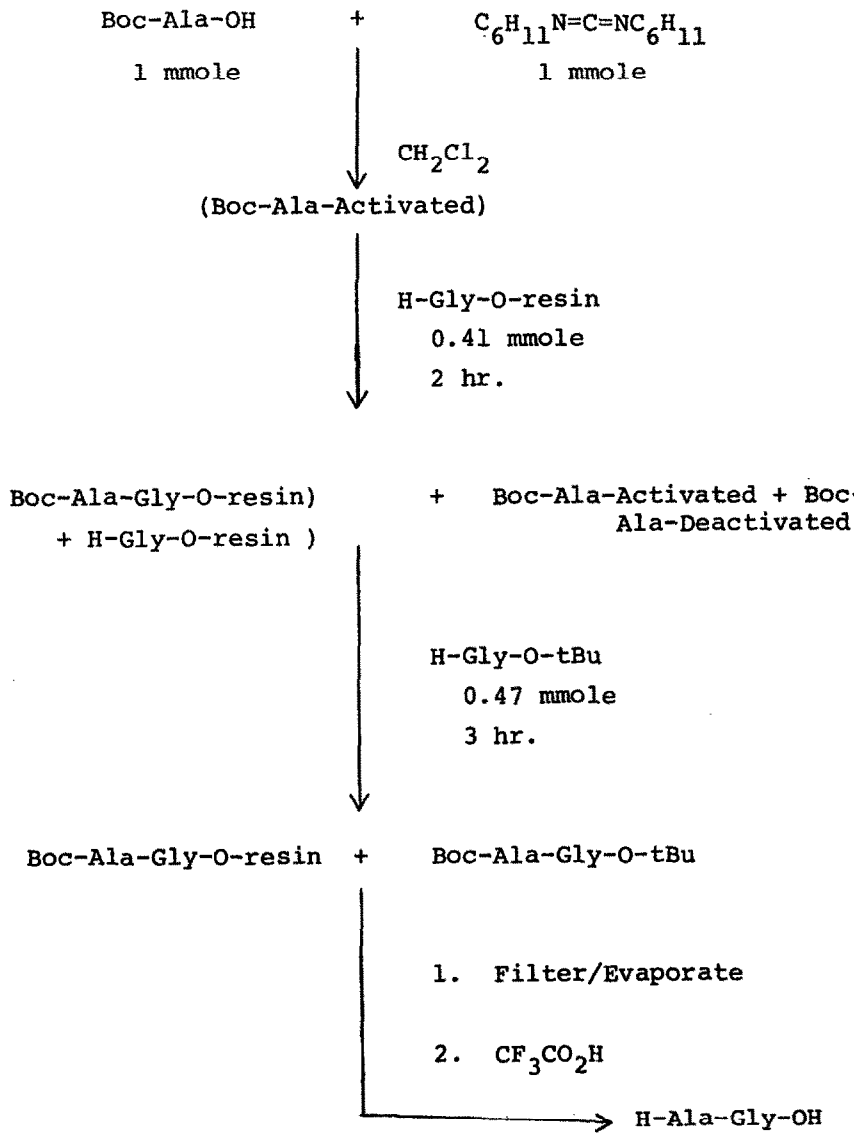
and a second nucleophile is added to the reaction after various time intervals. If reserve activated N-protected amino acid is still present, it will be intercepted by the nucleophile and the amount of solution product formed gives an indication of the reserve activated N-protected amino acid.

In the present study, 2 hr was arbitrarily chosen as time zero to add nucleophile H-Gly-O-tBu (0.47 mmole) to the reaction of carbodiimide-activated Boc-Ala-OH (1 mmole) and H-Gly-O-resin (0.41 mmole). Since it was shown in this run that acylation of H-Gly-O-resin was essentially complete, a maximum of 0.59 mmole of activated Boc-Ala-OH was left to react with 0.47 mmole of H-Gly-O-tBu. Chromatographic analysis showed that acylation of H-Gly-O-tBu, i.e., formation of Boc-Ala-Gly-O-tBu, was also essentially complete. Therefore, the reserve amount of carbodiimide-activated Boc-Ala-OH was at least 80% of the theoretical maximum after 2 hr. This value may be

SCHEME I



a minimum since the amount of nucleophile was less than the maximum potential amount of activated Boc-Ala-OH. The relative amounts of acylating reserve after 3, 5.3 and 8.5 hr were 74, 63 and 61%, respectively. Obviously, these three values are maxima. These results demonstrate that substantial availability of carbodiimide activated

N-protected amino acids may be present in SPPS after periods of up to ca. 8 hr, contrary to some previous views. This data might appear to be useful in extending the time of some sluggish solid phase coupling reactions, but as we shall discuss later, this may not be a good practice.

We observed, in another coupling experiment of carbodiimide-activated Boc-Ala-OH with H-Gly-O-resin, that an infrared scan of the reaction solution 3 hr after coupling was initiated had characteristic absorption bands for N-urethane protected amino acid anhydride (1750 and 1825 cm^{-1}) and dicyclohexylcarbodiimide (2120 cm^{-1}) (9). This observation indicates that the activated specie in the reaction was the symmetrical anhydride of Boc-Ala-OH, which is in agreement with the recent work of Rebek and Feitler (10). Furthermore, this observation is also consistent with the work of De Tar, et al (9), who found that equal molar amounts of carbobenzyloxyglycine (Z-Gly-OH) and DCCI produced a half mole of $(Z\text{-}Gly)_2O$ leaving half a mole of DCCI unchanged. On standing in the presence of the excess DCCI, the anhydride proved to be unstable. Thus, loss of available activated Boc-Ala-OH with time in this study might best be attributable to the lability of the corresponding anhydride in the presence of excess DCCI. For this reason, Rebek and Feitler suggest using a 2:1 Boc-amino acid: DCCI stoichiometry or preformed purified anhydride in SPPS. Recently, Merrifield, et al. (11) in a very careful study showed that symmetrical anhydrides of N-urethane protected amino acids have the potential for rearrangement, particularly at room temperature for long time periods, which could lead to the incorporation of dipeptide units during SPPS. This side reaction is negligible during 2-hr carbodiimide-mediated SPPS coupling reactions, but could become significant during long coupling periods up to 24 hr. This side reaction would conceivably be enhanced by excess carbodiimide which turns out to be the case in 1:1 Boc-amino acid: DCCI stoichiometry. Indeed, re-inspection of the chromatograms of

this study for H-Ala-Gly-OH did show some extraneous peaks near the H-Ala-Gly-OH peak of the long (5.3 and 8.5 hr runs) runs. Thus, all factors considered it would be best to run all solid phase peptide reactions for no more than two hr even though substantial acylating power may persist for much longer. For sluggish couplings then, multiple 2-hr acylations should be employed.

This method of determining the availability of carbodiimide activated N-protected amino acids in SPPS should be readily adaptable to other amino acids, since all of the reagents and materials, including H-AA-Gly-OH dipeptides, are readily available.

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