DETERMINING THE AVAILABILITY OF ACTIVATED AMINO ACIDS FOR SOLID PHASE PEPTIDE SYNTHESIS

Andrew M. Tometsko

Department of Biochemistry

University of Rochester Medical Center

Rochester, New York 14642

Received December 21,1972

Summary. Data concerning the reaction characteristics of each amino acid are of fundamental importance for providing optimum reaction conditions and high yields during polypeptide synthesis. Loss of activated amino acid as a function of time would have a pronounced effect on the efficiency of synthesis. An assay procedure is described which quantitatively determines the availability of DCC activated amino acids as a function of preincubation time. The assay was employed to follow the inactivation of t-BOC.Leu(DCC) and indicated a significant loss of activated amino acid.

N,N'Dicyclohexylcarbodiimide (DCC) (1) has been used extensively as an amino acid activating agent during conventional(2,3,4) and solid phase(5,6,7) polypeptide synthesis. The mechanism of amino acid activation (Figure 1) involves the formation of an O-acylisourea derivative(I) which subsequently reacts with an amine to form a peptide bond(II) and dicyclohexylurea(III). Reaction times from 2-24 hours have been employed for DCC mediated coupling reactions. However, the underlying assumption that the activated amino acids are stable for long periods of time has not been experimentally verified. A major side reaction of DCC activated amino acids involves the formation of N-acylurea derivatives(NAU)(IV) as a result of an intramolecular rearrangement (8), resulting in the loss of activated amino acid and a corresponding decrease in peptide bond formation. Similarly, solvent impurities could effectively

reduce the amount of activated amino acid available for coupling. As discussed previously(9,10),data reflecting the life time of the activated amino acids are of fundamental importance for providing optimum reaction conditions for each amino acid during automated peptide synthesis.

This communication describes a facile method for assaying the availability of activated amino acids, and employs the assay to follow the loss of DCC activated t-butyloxycarbonyl-L-leucine (t-BOC.Leu) as a function of time.

MATERIALS AND METHODS

1) Stock solution A contained t-BOC.Leu(250 mg.;1.07 mmoles) and triethylamine (0.2 ml.) in methylene chloride(25 ml.).

2) Stock solution B contained dicyclohexylcarbodiimide(302 mg.;1.47 mmoles) in methylene chloride(25 ml.).

3) Stock solution C contained saturated sodium methoxide in ethanol. A sample(10 ml.) was diluted to 25 ml. and was titrated with standard HCl giving an alkaline concentration of 10 meq./ml.

4) t-BOC.Ala resin was prepared by the method of Merrifield (11), and contained 0.250 meq/gm.

Assay Procedure. Initially (t=0), stock solutions A and B were combined. Samples of the preincubation solution (3 ml.) were removed at specified times (t= 0, 2, 4, 6, and 8 hours). Each sample was transferred to a vial (4 ml.) containing deprotected alanine resin (25 mg.) and methylene chloride (1 ml.). The vials were capped and were placed on an inverting shaker for 24 hours at 25 C. The resin in each vial was then filtered with a coarse

sintered-glass funnel (2 ml.), was washed with methylene chloride, and was dried.

Alanine and the dipeptide (t-BOC.Leu.Ala) were removed from the resin by saponification with a sodium methoxide-ethanol solution, modifying a method introduced by Loffet(12). Specifically, resin samples(10 mg.) were weighed (± 0.1 mg.) into collection vials(4 ml.). Following the addition of solution C (3 ml.), each vial was capped and placed on an inverting shaker for one hour. The resin samples were then filtered through a disposable pipette equipped with a plug of glass wool. Each sample was washed with ethanol once (1 ml.),concentrated acetic acid was added(3 drops), and the solvent was removed by evaporation with a stream of air. Each residue was then dissolved in pH 2.2 citrate buffer(2 ml.), and the samples were subjected to amino acid analysis (Beckman 120C amino acid analyzer).

Normalizing Amino Acid Analysis Data. The concentration of BOC.Leu and DCC used in this study provided a t=0 alanine content of 0.051 μmoles/mg. of resin. (Total alanine content was 0.250 μmoles/mg of resin.) The mole fraction of alanine , α , for each preincubation time ,t, was determined as follows:

$$\alpha = \frac{C(t)-C(to)}{C(total)-C(to)}$$

where concentration ,C, is given in μmoles of alanine per mg. of resin, C(t) and C(to) indicate the alanine concentrations obtained at times, t and t=0, respectively, and C(total)

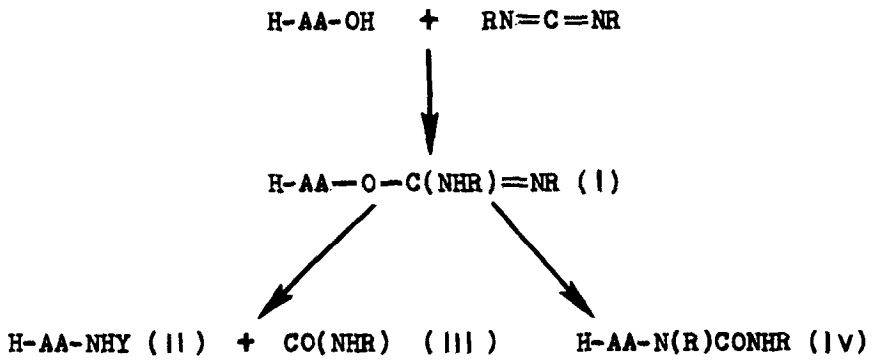

Figure 1. The reaction of an amino acid, AA, with DCC results in the formation of an acylisourea derivative, I, which could react with an amine to form a peptide bond (II) and the corresponding urea, or alternatively, could undergo an intramolecular rearrangement to the N-acylurea derivative (IV).

---

indicates the total alanine content of the resin. Thus, the values of α vary between 0.00 (initial alanine concentration with high coupling) and 1.00 (no coupling), for each sample. Plotting α as a function of preincubation time facilitates the comparison of data obtained with different initial and total alanine concentrations in different experiments.

RESULTS AND DISCUSSION

The efficiency of peptide synthesis depends upon the rates of coupling and inactivation of each activated amino acid. The coupling reaction will depend upon the reactivity of each amino acid, whereas the inactivation is a complex process that could involve side reactions, solvent impurities, temperature effects, etc.. Although the coupling characteristics of some amino acids have been reported (13), information concerning the inactivation process has been limited by the lack of a suitable assay method. In this report the inactivation profile of DCC activated t-BOC.Leu has been determined by preincubating the amino acid

with DCC for various times followed by incubation of the solution with alanine attached to a solid phase polymer. The amount of dipeptide and unreacted alanine obtained following the coupling reaction depends upon the amount of activated leucine initially present and the time of incubation. The quantitative determination of the alanine content of the resin sample as a function of preincubation time provides a profile of the inactivation process under the reaction conditions employed in this study. As shown in Figure 2, the fraction of unreacted alanine(α) increases as a function of preincubation , Under the

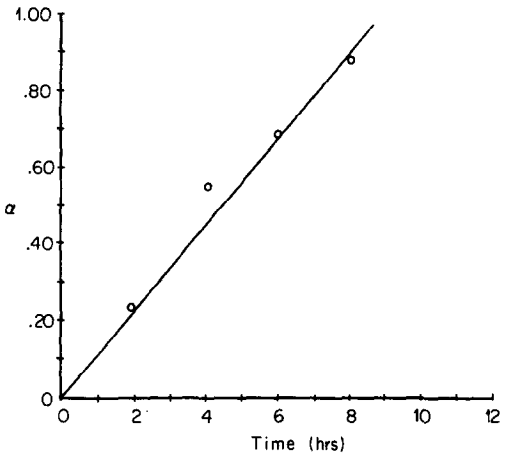

Figure 2. The mole fraction of alanine, α , is plotted as a function of preincubation time. Alanine concentration will vary between 0.00 (initial coupling level) and 1.00 (no coupling).

described reaction conditions , coupling with DCC activated BOC.Leu in excess of 4 hours would provide diminishing returns in terms of peptide bond formation. When coupling reactions have not reached completion during the initial two hour interval, it would be advisable to provide a second addition of activated leucine rather than extend the reaction time.

The observed increase in alanine content (decrease in

coupling) indicates that significant amounts of activated amino acid are being lost to inactivation processes. At some time during inactivation, the concentration of activated amino acid(C) will be one-half the original value (i.e. C/2). In order to compare the mole fraction of alanine obtained as a function of concentration, a parallel experiment was run in which alanine resin samples were coupled with activated BOC.Leu in concentration of C, C/2 and C/4 with no preincubation. The values of α obtained were 0, 0.46 and 0.85 respectively, indicating that an alanine concentration corresponding to C/2 occurs at about 4 hours.

As inactivation profiles of each amino acid become available, the reaction conditions can be altered to maximize the coupling yield.

REFERENCES

1. Sheehan, J. C., and Hess, G.P., J. Am. Chem. Soc. 77, 1067(1955).
2. Hofmann, K., and Katsoyannis, P.G., In "The Proteins", H. Neurath Ed., Academic, New York, N. Y., (1963).
3. Schröder, E., and Lubke, K., "The Peptides", Vol. I, translated by E. Gross, Academic, New York, N.Y.(1965).
4. Bodanszky, M., and Ondetti, M. A., "Peptide Synthesis", Interscience, New York, N.Y.(1966).
5. Merrifield, R. B., Advan. Enzymol., 32, 221(1969).
6. Stewart, J. M., and Young, J. D., "Solid Phase Peptide Synthesis", Freeman, San Fransisco, Calif.(1969).
7. Marshall, G. R., and Merrifield, R. B., In "Biochemical Aspects of Reactions on Solid Supports", G. R. Stark, Ed., Academic, New York, N. Y.(1971).
8. Khorana, H. G., Chem. Rev. 53, 145(1953).
9. Tometsko, A. M., Computers Biomed. Res. 5, 156(1972).
10. Tometsko, A. M., Garden, J., and Tischio, J., Rev. Sci. Instrum. 42, 331(1971).
11. Merrifield, R. B., J. Amer. Chem. Soc., 85, 2149(1963).
12. Loffet, A., Experientia 23, 406(1967).
13. Ragnarsson, U., Karlsson, S., Sandberg, B., Acta Chem. Scand. 25, 1487(1971).