Acylation Monitoring in Solid Phase Peptide Synthesis by the Equilibrium Distribution of Coloured Ions

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Spectrophotometric monitoring of the release of anionic dye from the support matrix during the acylation step in solid phase peptide synthesis (counterion distribution monitoring; CDM) is a sensitive indicator of the progress of this often problematic part of the process; this new method is readily incorporated into automated instrumentation where it permits feedback control of the time allowed for the coupling reaction, with consequent improvements in product quality and the speed of synthesis.

We report here a new monitoring method for solid phase peptide synthesis[1] which has significant advantages over those previously available. Counterion distribution monitoring (CDM) operates simultaneously with the acylation reaction and displays high sensitivity in the critical region of the reaction profile, where the reaction approaches completion. It is readily adaptable to automated instrumentation.

Present approaches either make use of the changing composition of the solution phase to infer the condition of the matrix-bound substrate, or are based upon quantitative or

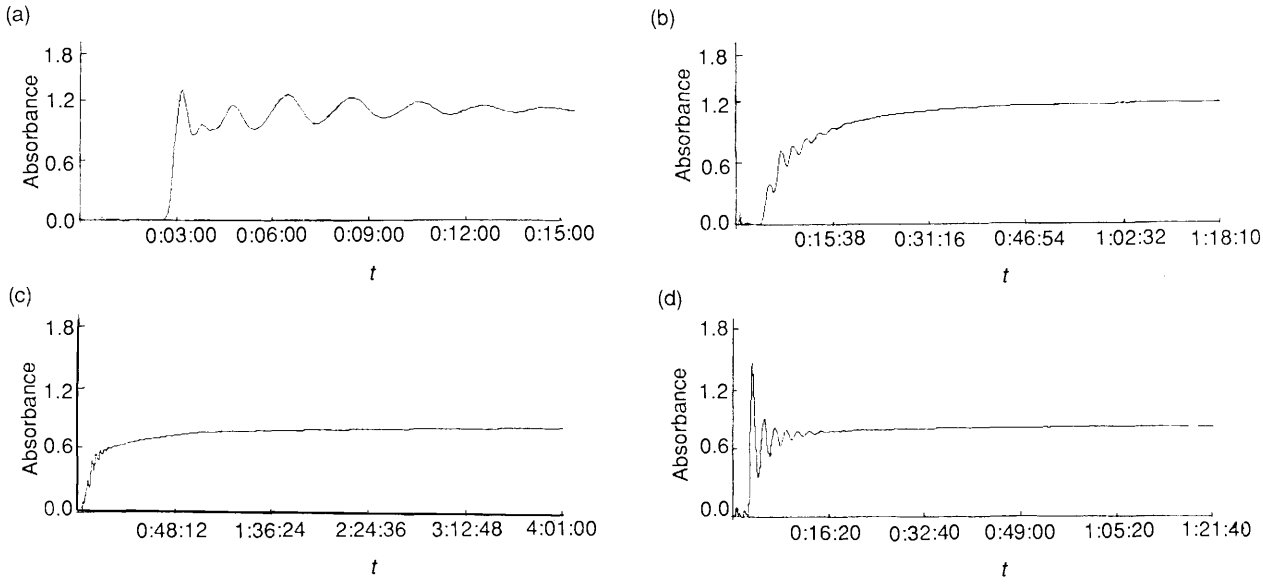

Figure 1. Profiles of absorbance at 439 nm of the reagent solution during selected acylations in the assembly of H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH. (a) Ala; (b) Ile; the coupling of Val (c) was incomplete after 4 h; at the end of this time the reagents were replaced (d) and the reaction allowed to continue for 16 h (only the initial portion of this profile is shown). The time is given in h: min: s.

qualitative analysis of amino groups remaining as acylation of the *N*-terminus proceeds. A major obstacle in the former case is the need to measure small changes in relatively large quantities, since changes in solution composition which accompany the final stages of reaction are necessarily minor. *N*(α)-Deprotection has been used effectively in the fluoren-9-ylmethoxycarbonyl (Fmoc) method[2] to confirm the success of the preceding acylation, but this method suffers from the combined disadvantages of insensitivity and the inability to retrieve the synthesis.

The most sensitive methods currently available for signalling incomplete couplings are of the second type.[3—8] They generally allow reacylation but require either additional reactions in the synthetic cycle to introduce and then remove a reporter reagent,[3,4] the withdrawal of a sample of the support for testing,[5—7] or spectrophotometric measurements on a portion of the resin.[8,9]

CDM combines the advantages of both approaches since it continuously monitors the total population of amino functions on the support matrix using the distribution of a minute quantity of a reporter dye between these groups and cations in solution. It can be applied generally to synthesis protocols which use polar media and are acidic due either to added catalysts such as *N*-1-hydroxybenzotriazole (HOBt),[10] or to the products of reaction of activated amino-acid derivatives. As the coupling reaction proceeds the number of cationic sites on the support matrix due to protonated *N*-terminal amino groups decreases and the number of anions bound as counterions is reduced proportionately. An inert anionic dye introduced into the system is distributed between all available cations and, as the acylation proceeds, is progressively displaced from the solid phase into solution. The absorbance of the solution will therefore rise to a predictable maximum value when reaction is complete.

This method is in principle very sensitive since the amounts of dye and of dissolved cations in the system are under experimental control and can in molar terms be very small compared to that of amino groups present at the start of the acylation. A simple model predicts that, given equal affinity of the dye for the protonated *N*-terminal amino groups and for

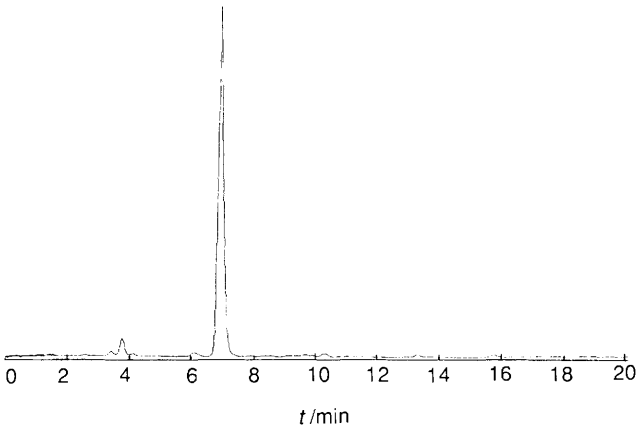

Figure 2. Chromatogram of the crude ACP (64—75), obtained after treatment of the synthesis support with trifluoracetic acid and subsequent solvent extraction of the residue after evaporation according to standard methods.

cations in solution, the dye will be equally distributed when the amounts of these are similar. In a hypothetical case where 0.04 equivalents of dissolved cation are present relative to the support functionality, half-maximal absorbance would correspond to 96% compelete reaction. In practice, using this ratio with 0.003 equivalents of dye present, we have found that residual affinity of the dye for other components of the reaction mixture reduces the sensitivity; but the impact on the effectiveness of the monitoring is not serious.

In contrast to approaches employing amino group quantitation by reversible covalent attachment of reporter reagents,[3,4] CDM operates in real-time. It depends upon ionic interactions and not on the reaction of sites already unreactive under the conditions of chain extension. Other methods using 3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one[8] and Bromophenol Blue[9] as acid–base indicators, whose conjugate bases are coloured when in proximity to the unacylated solid phase but are colourless as their neutral acids when displaced into the

acidic solution, share this advantage but require translucent synthesis supports and specially constructed photometers.

CDM, as far as its use has been explored, is applicable to a number of techniques for carboxyl group activation, provided that the reaction mixture is sufficiently acidic for significant protonation of amino groups. Suitable methods have been found to include: active ester–HOBt combinations and reaction *in situ* with carbodiimide–HOBt. CDM is compatible with a variety of support matrices† and can use any of a number of dyes which are both inert and have sufficient anionic character for them to bind to the amino groups of the substrate.

We have employed CDM in the synthesis of a number of peptides by the Fmoc method.‡ Small quantities of dilute solutions of various suitable dye–cation mixtures were introduced from a reagent reservoir. The dye distribution is simply followed by recirculating the reagent solution through a spectrophotometric flowcell. Feedback control is allowed by continuous measurement of the absorbance of this solution. The data obtained is interpreted to establish when each coupling is, for practical purposes, complete. In the case of rapid acylations which are effectively complete before the reagents in the circulating solution are fully mixed, the fluctuations in absorbance can be averaged to interpret an endpoint [Figure 1(a)].‡ Spectrophotometer traces for typical coupling reactions are shown in Figure 1: the *E. coli* acyl carrier protein fragment (amino acids 65—74) was chosen as an example here for comparison with data in the literature.[11] A chromatogram of the product is shown in Figure 2.§

Compared to coupling steps of standard, arbitrary durations there was a trend towards shorter overall synthesis times with increased homogeneity of the products.

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11 See *e.g.*: E. Atherton, L. R. Cameron, and R. C. Sheppard, *Tetrahedron*, 1988, **44**, 843.

† Ultrosyn (Pharmacia LKB), Polyhipe AM 450, MPM 250, and MPM 500 (National Starch), TentaGel (Rapp Polymere), and Sasrin (Bachem).

‡ All syntheses were carried out using a Pharmacia LKB model 4170 peptide synthesiser modified to include operation program changes, connection of a dye loading loop, and a 10 mm spectrophotometric flowcell into the recirculation loop. The synthesis employed 10^{-4} equivalents of polyacrylamide–kieselguhr support ('Ultrosyn A'; Pharmacia LKB), with the carboxyl terminal glycine residue attached to the hydroxymethyl phenoxyacetamide linker using standard methods. 5×10^{-7} mol of the Fmoc-amino acid pentafluorophenyl ester and of HOBt were used at each peptide coupling. The dye and cation were respectively Quinoline Yellow, water soluble (3×10^{-7} mol) and di-isopropylethylamine (4×10^{-6} mol), protonated in the acidic medium, introduced together in dimethylformamide (1 ml).

§ Column: ODS2, 4 × 250 mm (Pharmacia LKB). Mobile phase: A, trifluoroacetic acid–water (0.1% v/v); B, trifluoroacetic acid–acetonitrile (0.1% v/v); 0—100% B in 30 min; flow rate 1.0 ml min^{-1}. Sample: 0.02 mg in solution A (0.02 ml). Detection wavelength 215 nm.