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Serine Decomposition in Solid-State Catalytic Isotope Exchange of a Peptide

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In connection with work related to cAMP-dependent protein kinase, a tritiated substrate, Arg–Arg–Ala–Ser–Val–Ala, was prepared from the corresponding unlabeled peptide by solid-state catalytic isotope exchange. Varying amounts of side products with chromatographic properties very similar to those of the substrate were observed. Careful chromatographic comparison with a complete set of diastereomers eliminated racemization as the reason for this heterogeneity. Instead, the serine residue was identified as the major source of by-products, presumably due to primary elimination of water followed by saturation of the dehydroalanine residue, transforming it into a racemic alanine, thus giving rise to Arg–Arg–Ala–(D, L–Ala)–Val–Ala.

INTRODUCTION

Tritiated compounds are of exceptional importance as tracers in modern bioscience and various procedures have been developed for the preparation of such compounds (*1*, *2*). One of these involves isotope exchange reactions between substrates and tritium gas and is of particular interest for the labeling of compounds of high molecular weight, including peptides.

A new procedure, called solid-state catalytic isotope exchange (SSCIE), has been introduced to accomplish fast and efficient isotope exchange reactions and has recently been applied to peptides (*3*, *4*). Using this method, biologically active peptides multiply labeled with isotopes of hydrogen have been obtained. Normally in this procedure there is a strong preference for hydrogen exchange at the α-carbon and as the reaction proceeds essentially complete substitution of this hydrogen can be obtained before side chain substitution becomes prominent (*4*).

In connection with other work now in progress, we required a tritiated peptide, Arg–Arg–Ala–Ser–Val–Ala, as a substrate for cAMP-dependent protein kinase (*5*), and such a sample was obtained by the SSCIE method. Although the tritiated peptide could be efficiently purified by HPLC, we noticed that the crude product

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occasionally contained considerable amounts of impurities with properties very similar to those of the target molecule. The tritiation procedure requires a rather high temperature, normally 160°C, in order to secure a highly labeled product and we noticed that the amounts of impurities also increased with temperature. As the hydrogen exchange takes place at asymmetrical carbon atoms, our first suspicion in this context was that racemization might have occured. Due to the fact that we already had access to a complete set of its diastereomers (*5d*) as well as experience with separation and analysis of such peptides by HPLC (*6*), we set out to attempt to identify the major impurities formed by this technique. Although our original hypothesis about racemization turned out to be wrong, during this investigation a major, and in retrospect more plausible, side reaction of serine peptides was identified, the evidence for which will be discussed in this report.

MATERIAL AND METHODS

The peptides were synthesized by the solid phase method of Merrifield (*7*) and were purified and analyzed as described previously (*5d*). Three peptides, Arg–Arg–Ala–Xxx–Val–Ala, Xxx = L-Ala, D-Ala, or Gly, were made in the same way explicitly for this study.

Tritiated samples were prepared by the method of SSCIE (*8*). As a catalyst, 5% $Pd/BaSO_4$ (Fluka) was used. Lyophilized mixtures of peptide, rhodium chloride, and catalyst (in the ratio 1 : 1 : 5) were exposed to tritium gas, normally at 160°C, but in a few cases even at 200°C, for 1 h at a pressure of 300 mm Hg. After removal of labile tritium with 50% ethanol, the crude product was purified by ion-pair chromatography on a column (250 × 4.6 mm i.d.) containing Zorbax C_{18}, 5 μm, using a gradient of solvent A (50 mM $NH_4H_2PO_4$, pH 3.0, containing 5 mM of sodium 1-pentane sulfonate) against solvent B (CH_3CN) at a flow-rate of 1 ml/min at a rate of 1% B/min starting from 0% of B. In the chromatography of tritiated peptides, fractions of 10 or 12 drops were collected and counted for radioactivity using a Philips P.W. liquid scintillation counter. In model experiments, hydrogen containing 0.1% of tritium was used. Tritiated samples were stored in 80% methanol solution at −15°C.

The chromatographic equipment used for analytical purposes was identical to that described previously (*6b*). The column (200 × 4.6 mm i.d.) contained Spherisorb C_8 (5 μm, 97,500 plates/m) and gave better selectivity than the corresponding C_{18} packing material (*6a*). The reagents used for the preparation of buffers were the same as in the study of the pentapeptides quoted above (*6b*). Thus, sodium 1-pentane sulfonate was applied for ion-pair formation in phosphate buffers containing ethanol as organic modifier. Further details are given in the legends to the figures.

Before amino acid analysis, peptide samples were hydrolyzed for 24 h in 6 N HCl containing phenol (1 g/liter). The analyses were performed in the Amino Acid Analysis Laboratory, Biomedical Center, Uppsala.

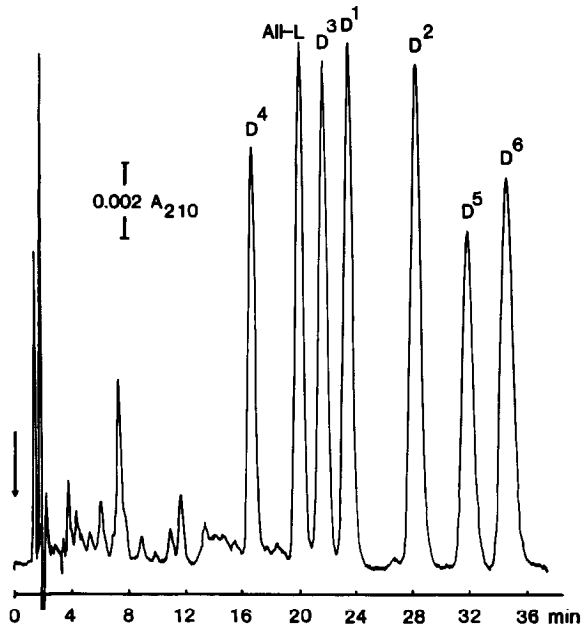

FIG. 1. Separation of a set of hexapeptide diastereomers related to all-L-Arg-Arg-Ala-Ser-Val-Ala on a C_8 column. Support: Spherisorb C_8, 5 μm. Mobile phase: 0.1 M phosphate buffer, pH 3.0/ethanol, 90.2/9.8. Counterion: 0.015 M 1-pentane sulfonate. Flow-rate: 1.0 ml/min. Detection wavelength: 210 nm. Temperature: 25°C.

RESULTS AND DISCUSSION

Figure 1 demonstrates that complete baseline separation of all seven peptides belonging to this set of diastereomers was accomplished. The established conditions were also applied to check the purity of the crude all-L-hexapeptide after SSCIE at 160°C as well as under more drastic conditions. Figure 2A depicts a chromatogram of a product obtained at 200°C and shows that treatment of the peptide with hydrogen under the conditions of SSCIE results in partial degradation, leading to the appearance of several peaks in the same region. However, a careful comparison, which included cochromatography with authentic diastereomers, failed to indicate identity between the latter ones and the degradation products (results not shown). On the contrary, we concluded that no significant racemization had taken place even under the more drastic conditions used.

At this stage amino acid analyses of two different crude samples from SSCIE experiments at 200°C were carried out: Arg, 1.91 and 1.89; Orn, 0.04 (both); Ala, 2.45 and 2.65; Ser, 0.43 and 0.27; Gly, 0.12 (both); and Val, 1.08 and 1.07. These results show that the decomposition of serine is accompanied by an increase in alanine and that some glycine is also formed. Consequently, the syntheses of three additional peptides were undertaken (see Materials and

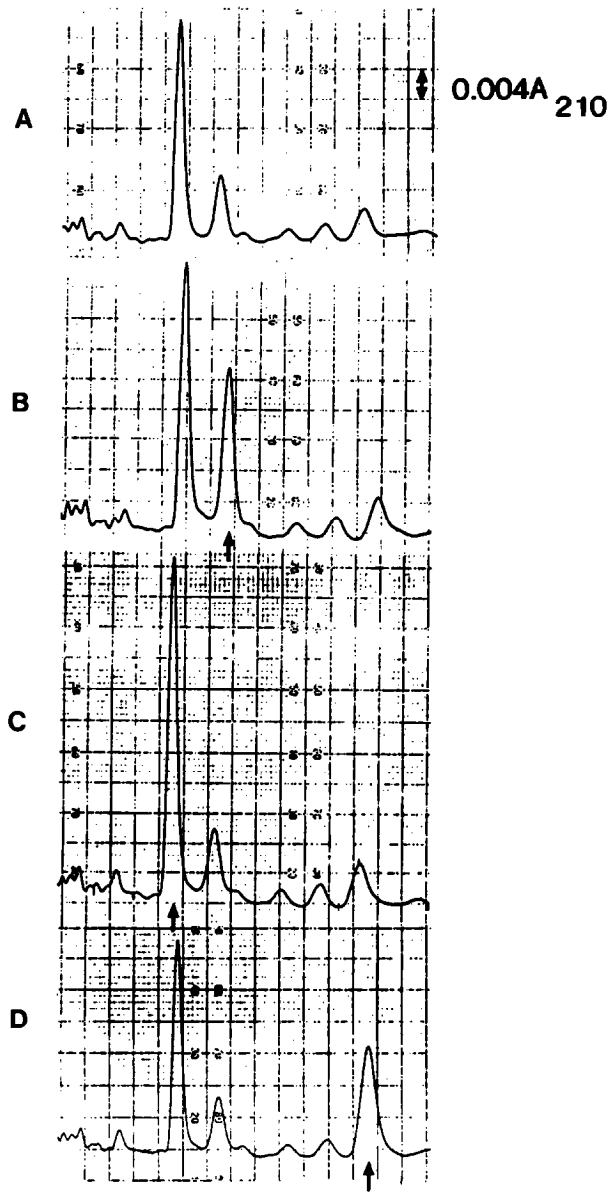

FIG. 2. Chromatogram of all-L-Arg–Arg–Ala–Ser–Val–Ala after SSCIE with hydrogen at 200°C. For chromatographic conditions, see the legend to Fig. 1. A, crude product; B–D, cochromatography with authentic [L-Ala^4]-, [Gly^4]- and [D-Ala^4]-analogs, respectively.

Methods). The results of cochromatography of the three latter peptides and one of these crude products are shown in Figs. 2B–2D. In addition, some decomposition of arginine residues was noticed, but this phenomenon was not studied further.

The fact that on SSCIE involving amino acids and peptides the initial substitution takes place at the chiral carbon atoms (*4*, *8*) warrants careful attention to the possibility of racemization. Our recent success with respect to the chromatographic separation of diastereomers of a similar peptide (*6b*) prompted us to apply an identical approach here. The excellent separation obtained also in this case after optimization would easily have allowed detection and reliable quantification of diastereomers on the order of 1%, if present. The absence, after SSCIE, of chromatographic peaks that could be identified as diastereomers of the all-L hexapeptide means that this method can now be used with more confidence for tritiation of biologically active peptides.

The need for the application of temperatures above 140°C (*8*) in order to accomplish high-level labeling of peptides by SSCIE proved to have undesirable consequences in this case. Obviously, so far the influence of temperature on the stability of peptides has not been systematically studied in solution nor in the solid state. Nevertheless, there are a few references to amino acids with some relevance in this context. Wieland and Wirth (*9*) found that, upon heating in $Ba(OH)_2$ solution, serine gave rise to glycine and alanine, presumably through initially formed dehydroalanine. Retroaldol cleavage should result in glycine, whereas hydrolysis through pyruvate and transamination was suggested to have produced alanine in this case. Vallentyne (*10*) noticed that serine was among the most unstable amino acids when heated in water in the absence of oxygen in sealed tubes and recorded dramatic effects of temperature on the decomposition rate in the range 152–216°C. Glycine and alanine were formed together with ammonia and ethanolamine.

In light of the studies on free serine quoted above, our results can be rationalized as follows. Upon heating at a temperature of 160°C or higher the peptide all-L-Arg–Arg–Ala–Ser–Val–Ala partly undergoes elimination of water at the serine residue, thereby giving rise to the corresponding dehydroalanine peptide. For simplicity, this side reaction was studied at 200°C. Part of the intermediary product, in the presence of the palladium catalyst, then adds isotopic hydrogen, as a result of which the two L- and D-alanine peptides are formed. In addition, part of the dehydroalanine peptide is postulated to undergo retroaldol cleavage to give the corresponding glycine peptide, the presence of which was not strictly proven, but is rather inferred from the amino acid analyses.

Other aspects of stability and decomposition of peptides under SSCIE conditions will be described elsewhere (*11*).

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