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REVERSIBLE MODIFICATION OF ARGININE RESIDUES WITH GLYOXAL

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SUMMARY. Glyoxal reacts with arginine to yield several ninhydrin reactive adducts. One of these, the product favored in strongly acidic solutions, decomposes in the presence of o-phenylenediamine with regeneration of free arginine. Reversible modification of an arginine residue in a peptide has been demonstrated. The conditions for reversibly blocking the guanidino group with glyoxal differ in some respects from those reported for reversible blocking of arginine sidechains with cyclohexanedione. It appears that the two dicarbonyl reagents, glyoxal and cyclohexanedione, might be complementary in their applications to reversible protein modification and to polypeptide semisynthesis.

INTRODUCTION. Glyoxal[1] and other 1,2-dicarbonyl compounds[2-7] react with the guanidino function of arginine. Selective modification of arginine residues of polypeptides and proteins with dione reagents is commonly applied to structure-activity studies[5,8-12] and also for restriction of tryptic hydrolysis of peptide chains[3,5,13]. Specificity for the guanidino group and the particular types of products formed both depend upon reaction conditions as well as upon the particular dicarbonyl reagent used.

Patthy and Smith[14] have reported conditions for reversible blocking of arginine sidechains with cyclohexanedione. The single product formed by treatment of arginine with cyclohexanedione in weakly alkaline borate buffers, N^7,N^8-(1,2-dihydroxycyclohex-1,2-yl)-arginine, decomposes in the presence of neutral solutions of hydroxylamine with regeneration of the guanidino function. The recovery of arginine or arginine peptides from this type of adduct with cyclohexanedione or cyclohexanedione derivatives can also be accomplished with other types of dione-trapping agents such as o-phenylenediamine[15].

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We have found that of the several adducts which may be formed by treatment of arginine with glyoxal, one particular adduct (I) decomposes in the presence of o-phenylenediamine at pH greater than 8 with regeneration of free arginine. The labile adduct is the sole ninhydrin-positive product formed either by treatment of arginine with glyoxal in concentrated HCl[1] or by treatment of Cbz-arginine with glyoxal at pH 8.1 followed by removal of the Cbz group with HBr in acetic acid[9]. It is also the major product obtained by treatment of arginine with glyoxal in borate buffers. The lability of the adduct to o-phenylenediamine, along with its stabilization by borate ions, point toward an N^7,N^8-(1,2-dihydroxyeth-1,2-yl)-arginine structure.

The multiple adducts of glyoxal with arginine and their relative reactivities toward o-phenylenediamine carry over into studies on an arginine containing peptide derivative. An arginine containing peptide derivative was treated with glyoxal at pH 8.1 to yield a product in which at least 80% of the arginine residues were modified. Treatment of this material directly with o-phenylenediamine did not reverse the modification of arginine, but when the peptide derivative was treated first with trifluoroacetic acid, then with o-phenylenediamine solutions, the arginine content of the peptide was completely recovered.

Some arginine derivatives unstable to mildly alkaline conditions can be blocked with glyoxal in acidic media and not with cyclohexanedione in borate buffers. For example, Cbz-Arg-ONP salts decompose rapidly upon exposure to alkaline buffers, but the glyoxal adduct is easily prepared by brief treatment with glyoxal in concentrated HCl.

Specific differences in conditions for reversible modification of arginine residues may make either glyoxal or cyclohexanedione more suitable for differential and independent protection of multiple arginine residues during polypeptide semisynthesis.

EXPERIMENTAL. The acid stable adduct I was prepared by two routes. In the first method, after Kotai et al[9], Cbz-arginine was treated with aqueous glyoxal at pH 8.1, then with HBr in glacial acetic acid. The second method of synthesis is essentially that of Bowes and Cater[1]. Arginine hydrochloride (200 mg) was dissolved in 10 ml 12 M HCl along with 1 ml 40% aqueous glyoxal. After 24 hr the reaction mixture was evaporated under reduced pressure without heating above 40°. The product was twice redissolved in small volumes of water and evaporated to dryness, then it was taken up in 15 ml water and stored as a frozen solution.

Chromatographic analysis of ninhydrin-positive compounds was carried out with the basic amino acid analysis system of a Beckman-Spinco Model 120C amino acid analyzer, essentially the chromatographic technique used by Bowes and Cater. The position of adduct I in such chromatograms is very close to that of ornithine. Both methods of preparation of the arginine-glyoxal adduct gave single ninhydrin-positive products which were indistinguishable either in the column chromatography, by high voltage electrophoresis, or in subsequent chemical studies.

Although the treatment of arginine with glyoxal in 12 M HCl was routinely left for 24 hr, the reaction is complete within minutes. In one experiment samples of the reaction mixture were withdrawn at timed intervals after addition of the glyoxal and diluted directly in citrate buffers for chromatographic analysis. In the first sample, withdrawn 10 min after the addition of glyoxal, arginine had already disappeared completely from the solution and adduct I had appeared as the sole product.

Treatment of arginine with glyoxal at pH 8-9 in 0.2 M borate buffers also yields primarily adduct I rather than the mixture of products normally produced in this pH range in the absence of borate. There were, however, small amounts of other products in these mixtures and the borate buffers were not easily removed. This method of making adduct I was not used for preparative purposes, but only to demonstrate the stabilizing effect of borate ions on this particular adduct.

Solutions of the arginine-glyoxal adduct (approximately 0.07 M, based upon the quantitative conversion of arginine or Cbz-arginine to I) from both preparative synthetic routes were adjusted to pH values ranging from 6 to 11.5. Samples of these incubates were withdrawn at timed intervals and analyzed for ninhydrin-reactive components. At pH 6 and at pH 7 there was no change in the concentration of the starting material over 20 hr, nor did additional ninhydrin-positive materials appear in ion exchange chromatograms. At pH values of 8-11.5 adduct I disappeared over a period of hours and two ninhydrin-reactive compounds with earlier elution times appeared. After 24 hr or longer, depending upon the pH, the original acid stable adduct had disappeared and been replaced with a mixture of products characteristic of the direct treatment of arginine with glyoxal at the respective pH. At pH 11.5 a small amount of arginine, in addition to the two other major decomposition products, was present after incubation overnight.

Samples of I were diluted with 4 volumes of 0.2 M o-phenylenediamine dihydrochloride, the pH's of the resultant solutions adjusted to values ranging from 7 to 10. At timed intervals these solutions were centrifuged to remove a yellow precipitate, and aliquots of the centrifugal supernatant solutions were withdrawn for chromatographic analysis. At pH values of 8 and higher the starting material disappeared over a period of hours. In all cases the major product of the decomposition was arginine, but at pH 9 and pH 10 there were traces of other ninhydrin-positive products. The recovery of arginine was more rapid at the higher pH values, but the conversion to arginine without byproducts was achieved in the pH range between 8 and 9. At pH 8.1-8.3 only arginine was produced and the reactions were complete in 18-24 hr.

The synthetic peptide Bis(benzyloxycarbonyl-L-arginyl-L-asparaginyl)-L cystinyl-Bis(L-prolyl-L-leucylglycinamide)[16] was treated with aqueous

glyoxal at pH 8.1 and the product freed of excess reagent by gel filtration. An acid hydrolyzed sample of this product contained about 20% of the original arginine content. Since acid hydrolysis of the adduct I causes partial conversion to free arginine, the extent of blocking at the arginine residues was 80-100%. A portion of the glyoxal-modified peptide was treated with 0.2 M o-phenylenediamine at pH 8.2. After gel filtration a sample of this product was again hydrolyzed and subjected to amino acid analysis. The arginine content of the hydrolysates was unchanged by the phenylenediamine treatment. The peptide-glyoxal derivative was then dissolved in trifluoroacetic acid for 40 min and then retreated with phenylenediamine at pH 8.2 overnight. Peptide was again recovered by gel filtration and a sample hydrolyzed for amino acid analysis. The arginine content of the peptide was now returned to its full original ratio to the other amino acids, as determined by amino acid analysis before the original treatment with glyoxal.

The rapid reaction of glyoxal with arginine in 12 M HCl suggested that even moderately acid labile compounds might be able to survive the conditions required for glyoxal modification of the guanidino group. Cbz-Arg-ONp HNO_3 [18] (870 mg) was dissolved in 0.9 ml dimethylformamide. Aqueous glyoxal (0.3 ml 40% aqueous solution) was added to the mixture, then 9 ml 12 M HCl was added. An oil formed upon addition of the HCl solution, then redissolved after a few minutes. The reaction mixture was treated with 90 g ice as soon as the oil redissolved, and the precipitated product was ground in the cold aqueous solution until it was a loose white powder. The solid product was collected by centrifugation, washed with cold water, and recovered as a white powder (710 mg) by lyophilization of the frozen centrifugal pellet. Elemental analyses (C,H,N) were in agreement with a 1:1 molar adduct of Cbz-Arg-ONp and glyoxal, isolated as the hydrochloride salt half hydrate.

DISCUSSION. The reversible addition of glyoxal to the sidechain of arginine is, in some respects, similar to the reversible blocking of arginine residues with cyclohexanedione. Each reagent forms a variety of ninhydrin-positive adducts, one of which decomposes in weakly alkaline solutions in the presence of a scavenger for the dicarbonyl component. By appropriate control of reaction conditions it is possible to make the reversible adduct the sole product of the reaction between the guanidino compound and the dicarbonyl reagent. The stabilizing effect of borate ion in each case is consistent with a 1,2-diol group in the adduct. Further evidence for that structure has been reported in the case of the cyclohexanedione compound[14].

Details of the reversible blocking of arginine residues with glyoxal and with cyclohexanedione are sufficiently different that one or the other reagent may be chosen for a given application in peptide or protein chemistry. There is also a possibility that the two reagents can be used to independently control chemical or enzymatic reactions at separate arginine residues in a single molecule or in separate fragments being joined in a

polypeptide semisynthetic operation. Reaction with guanidino groups in acid media is characteristic of glyoxal and does not occur with cyclohexanedione or water soluble cyclohexanedione derivatives. These differences in reactivities among the dione reagents along with differences in reactivities of the arginine adducts are being investigated further to determine whether independent manipulation of glyoxal and cyclohexanedione blocked residues is possible.

We have earlier reported model schemes for the use of trypsin-labile protecting groups for amino and carboxyl functions during peptide synthesis[16-19]. Improved methods for independent manipulation of arginine residues might greatly facilitate the applications of such blocking groups in semisynthesis of complex polypeptides.

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