Studies on the Chemical Modification of Arginine

I. THE REACTION OF 1,2-CYCLOHEXANEDIONE WITH ARGinine AND ARGINYL RESIDUES OF PROTEINS*

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SUMMARY

The guanido group of arginine condenses with 1,2-cyclohexanedione in alkaline aqueous medium to form a new α-amino acid. The product (1,2-cyclohexanedione-arginine) was shown by independent synthesis to be N-(4-carboxy-1,3-diazaspiro[4.4]non-2-ylidene)-L-ornithine. At hydroxide concentrations of 0.2 M or higher, this was the principal product, and the reaction with free arginine and with the arginyl residues of model proteins was completed in 3 hours. At lower base concentration or with salmine at high base concentrations, a heterogeneous mixture of ninhydrin-positive products was observed. The reaction appears to be specific for the guanido group. Peptide maps of tryptic digests of 1,2-cyclohexanedione-treated chains of hemoglobin showed blocking of hydrolysis at arginyl bonds with no change in positions of tryptic peptides not adjacent to arginyl residues.

Limiting the hydrolytic action of trypsin on a protein by chemical modification of either the lysyl or the arginyl bonds serves a two-fold purpose. If a protein contains a large number of these bonds, protecting either type from tryptic action may reduce the number of peptides released to a manageable level. Since the modified amino acid joins two sections of the protein that are tryptic peptides in digests of untreated protein, the fused peptides can be used to determine the ordering of some of these peptides in the protein. Several methods are available for blocking tryptic digestion of lysyl bonds by chemical modification of α-amino groups (2-9), but no method for the specific modification of arginyl residues has been available until recently. Benzil forms a stable addition product with arginine, and proteins treated with benzil are resistant to tryptic hydrolysis of arginyl bonds (10); however, the insolubility in water of benzil and of the product of its condensation with arginine is an impediment to its use in protein and amino acid chemistry. Other 1,2-diketones, therefore, were tested qualitatively with respect to solubility in water, rapidity of reaction with arginine, and homogeneity of reaction product. 1,2-Cyclohexanedione was found to have favorable properties. An investigation was therefore undertaken of the reaction of CHD with arginine, the structure of the product, and the effect of CHD treatment on proteins; and the results were briefly summarized in an earlier report (1).

EXPERIMENTAL PROCEDURE

Materials

1,2-Cyclohexanedione (J. T. Baker Chemical Company, Phillipsburg, New Jersey) was recrystallized from petroleum ether before use. The α and β chains of human adult hemoglobin were prepared by countercurrent distribution (14) or by carboxymethyl cellulose chromatography (15). Oxidized B chain of bovine insulin was purchased from Mann. Salmine was purchased from Sigma as protamine sulfate, Grade I, and trichloracetic acid-trypsin was purchased from Worthington. 1-Aminocyclopentanecarboxylic acid was purchased from Aldrich. Nε-Carbobenzoxy-L-ornithine was purchased from Mann. Nε-Formyl-L-ornithine was prepared by the method of Wolf et al. (16). The melting point was 197° (decomposed). Reagent grades of other chemical reagents and solvents were used.

Reaction between 1,2-Cyclohexanedione and L-Arginine

Solutions of 0.5, 0.2, and 0.05 M NaOH containing 0.05 M l-arginine and 0.06 M CHD were prepared and allowed to stand at room temperature (25°). Samples of these solutions were removed at various intervals of time into equal volumes of 20% acetic acid. Amino acid analyses of the acidified reaction mixtures were carried out by high voltage electrophoresis.1

1 The abbreviation used is: CHD, 1,2-cyclohexanedione.

2 Reports of other methods for modifying arginine have appeared as abstracts (11, 12). The work of King has been published (13) since this paper was submitted.

3 W. Dreyer and E. Bynum, unpublished procedure.
Preparation of N'-4-Oxo-1,3-diazaspiro[4.4]non-2-ylidene-L-ornithine (Compound III in Figs. 1 and 2)\(^1\)

From L-Arginine (II) and 1,2-Cyclohexanedione (I)

CHD (0.67 g) was added to 1.05 g of L-arginine monohydrochloride dissolved in 100 ml of 2 N NaOH, and the solution was allowed to stand overnight at room temperature (25\(^\circ\)C). In order to desalt the solution it was then applied to a column (4.5 x 30 cm) of AG 50W-X2 (H\(^+\) form) (Bio-Rad), and the column was washed with water and eluted with 1.0 N ammonia. Fractions that contained ninhydrin-positive material other than ammonia were collected and evaporated to dryness. The residue was taken up in 30 ml of water and adjusted to pH 2.0 with 2 N HCl. This solution was applied to a column (2 x 45 cm) of AG 50W-X2 (buffered at pH 5.27 with 0.2 M citrate buffer at 50\(^\circ\)C), and the column was eluted with 0.2 M citrate buffer, pH 5.27. Fractions containing the major product (Compound III), as identified by paper chromatography, were collected. The combined solution was desalted as described above and evaporated to dryness. The residue was taken up in 20 ml of 50% ethanol. Acetone was added until slight turbidity appeared, at which point the solution was placed in a refrigerator overnight. The crystalline precipitate which formed was collected by filtration, washed with ethanol-acetone-water, and dried under vacuum over P₂O⁵. Yield 0.7 g, m.p. 240\(^\circ\) (decomposed), [α]\(_D\) +18.2\(^\circ\) ± 0.5\(^\circ\) (c, 1, in 5 N HCl).

Calculated: C 53.71, H 7.51, N 20.88
Found: C 53.82, H 7.44, N 21.00

From 2-(Methylthio)-1,3-diazaspiro[4.4]non-1-en-4-one (VII in Fig. 2) and N²-Formyl-L-ornithine

1-Acetyl-2-thio-1,3-diazaspiro[4.4]nonane-2,4-dione (V)—1-Aminocyclopentanecarboxylic acid (IV) (8.0 g) and 8.0 g of ammonium thiocyanate were added to 36 ml of acetic anhydride and 4 ml of acetic acid. The mixture was slowly warmed until the solids dissolved. The solution was permitted to stand at room temperature for 2 hours and was then poured into 300 ml of water with stirring. The precipitate which formed was filtered and dried to obtain brown material which was recrystallized twice from 70% ethanol with the use of a small amount of charcoal. Yield, 3.7 g; m.p., 195\(^\circ\).

Calculated: C 50.94, H 5.70, N 13.20
Found: C 50.97, H 5.96, N 13.47

2-Thio-1,3-diazaspiro[4.4]nonane-2,4-dione (VI)—A suspension of 8.4 g of Compound V in 7 ml of 6 N HCl and 14 ml of methanol was refluxed for 1 hour. The reaction mixture was evaporated until a precipitate appeared. The precipitate was collected by suction and recrystallized from 80% ethanol. Yield, 2.4 g; m.p., 195-195.5\(^\circ\).

Calculated: C 49.40, H 5.92, N 16.46
Found: C 49.31, H 5.88, N 16.51

N²-(4-Oxo-1,3-diazaspiro[4.4]non-2-ylidene)-L-ornithine (III)—

* This compound was previously referred to as L-2-[(4-amino-4-carboxybutyl)limino]-5,5-cyclo tetramethylene-4-imidazolidinone (1).
Fig. 3. High voltage paper electrophoresis of arginine (1), CHD-arginine (2), and of the products of the reaction of CHD and arginine in 0.05 N NaOH (3), 0.2 N NaOH (4), and 0.5 N NaOH (5). Product A is CHD-arginine.

A solution of 3.7 g of Compound VII in 90 ml of ethylene glycol was mixed with a solution of 6.8 g of N-formyl-L-ornithine in 15 ml of water adjusted to pH 10.5 with NaOH. The mixture was divided into several portions, which were sealed in test tubes and heated at 110° for 20 hours. The reaction mixtures were combined and evaporated to 50 ml under reduced pressure, and 8.0 ml of 2.5 N HCl in 40% ethanol were added to the concentrate. After the solution was warmed at 60-70° for 2 hours, 100 ml of 2 N NaOH were added. The solution was desalted, and the major product was isolated as described for Compound III above. The product was dissolved in 40 ml of 50% ethanol, and ethanol was added until the solution became slightly turbid. After this preparation stood in a refrigerator overnight, a crystalline precipitate was collected by filtration and dried at 80° for 2 hours under vacuum. The yield was 3.5 g. After recrystallization from 70% ethanol, the melting point was 240° (decomposed); [α]D 20 +16.0° ± 0.5° (c, 1, in 5 N HCl).

C13H12O4N4
Calculated: C 53.71, H 7.93, N 20.88
Found: C 53.87, H 7.65, N 20.63

Reaction of 1,2-Cyclohexanedione with Proteins

The protein (30 to 40 mg) was dissolved in 5 ml of 0.2 N NaOH. A 10-fold excess of CHD over the calculated arginine content was added. The solution was kept at room temperature (25°) for a period of time that depended upon the experiment and was then neutralized with 1 N HCl. In the treatment of salmine, a 3-fold excess of CHD was used in three experiments with 15 ml of 0.05 N NaOH, 10 ml of 0.5 N NaOH, and 5 ml of 1.0 N NaOH. Treated hemoglobin chains were desalted by dialysis against water, and insulin B chain was desalted by gel filtration on a Bio-Gel P-2 column (1.5 X 100 cm) with 0.01 N ammonia as eluent. Salmine was desalted on a Sephadex G-25 column (1.5 X 100 cm) with 0.005 N HCl as eluent. The desalted preparations were lyophilized.

Hydrolysis of Proteins—Protein (1 mg) was treated under vacuum with 1 ml of constant boiling HCl at 110° for 20 hours.

Tryptic Digestion—CHD-treated proteins were digested with trypsin at pH 8.0 or 9.0 in a reaction cell described by Merigan, Dreyer, and Berger (8). Trichloracetic acid-trypsin was used in a weight ratio of 1:100 of enzyme to substrate.

Amino Acid Analysis by Electrophoresis—Amino acids were separated on filter paper by the one-dimensional high voltage
The papers were stained with cadmium-ninhydrin (17), the stained spots were eluted (methanol-water (50:50)), and the absorbances of the eluates were measured at 500 μm.

**Amino Acid Analysis by Column Chromatography**—An automatic amino acid analyzer (Phoenix Precision Company K-8000V) was used.

**Peptide Analysis of Tryptic Digests of Proteins**—Peptide maps were obtained with use of the solvent system described by Hanada and Rucknagel (18).

**RESULTS**

**Reaction of 1,2-Cyclohexanedione with l-Arginine**—Several ninhydrin-positive products were separated by high voltage electrophoresis of reaction mixtures in 0.05 M NaOH. One major and one minor product appeared in 0.2 and 0.5 M NaOH (Figs. 3 to 6). The major product, electrophoretic Component A, which was the only one isolated and purified, will henceforth be referred to as CHD-arginine.

**Properties of 1,2-Cyclohexanedione-arginine**—The isolation and properties of this compound were described above. It is a ninhydrin-positive compound which can be separated from the other amino acids of protein hydrolysates by high voltage electrophoresis (1). Although it migrates electrophoretically among the neutral amino acids, it is eluted with the basic amino acids in column chromatography. Special conditions were required to separate it from lysine and histidine (Fig. 7). It is unstable in strong alkali, being degraded into a heterogeneous mixture when heated in 2.0 M NaOH at 110°C for 16 hours. 1-Aminocyclopentanecarboxylic acid, ornithine, and citrulline were detected in the reaction mixture by means of high voltage electrophoresis. On the other hand, CHD-arginine is relatively stable under the usual conditions of acid hydrolysis of proteins. When CHD-treated insulin B chain was hydrolyzed with 6 N HCl at 110°C,
recoveries of CHD-arginine of 92, 84, 74, and 63% were obtained after 20, 40, 70, and 140 hours, respectively.

**Determination of Chemical Structure of 1,2-Cyclohexanedione-arginine**—Lempert and Lempert-Srétér (19) and Lempert-Srétér, Solt, and Lempert (20) have shown that the condensation of benzil with monosubstituted guanidines in strongly alkaline solution results in imidazolidione derivatives. Since the product of the reaction between benzil and L-arginine was found to be

$N^5$-(5-oxo-4,4-diphenyl-2-imidazolidinylidyne)-L-ornithine, the analogous structure, $N^5$-(4-oxo-1,3-diazaspiro[4.4]non-2-ylidene)-L-ornithine (III in Fig. 1) was postulated for CHD-arginine.

K. Toi and H. A. Itano, unpublished results. This compound was previously referred to as L-2-[(4-amino-4-carboxybutyli)-iminol]-5,5-diphenyl-4-imidazolidinone (1).
The formation of 1-aminocyclopentaneacarboxylic acid, ornithine, and citrulline by alkaline degradation was consistent with the postulated structure.

An authentic sample of Compound III was synthesized as shown in Fig. 2. 2-Thio-1,3-diazaspiro[4.4]nonane-2,4-dione (VI) was prepared by the method used by Carrington (21) for 2-thio-1,3-diazaspiro[4.5]decane-2,4-dione. 1-Aminocyclopentaneacarboxylic acid (IV) was allowed to react with ammonium thiocyanate in acetic anhydride, and 1-acetyl-2-thio-1,3-diazaspiro[4.4]nonane-2,4-dione (V) was hydrolyzed in acid to Compound VI. The 8-methylated compound (VII) of Compound VI was prepared by the use of methyl iodide and KOH (22). The final product (III) was obtained by aminolysis of Compound VII (23) with N-formylornithine, followed by acid hydrolysis. Compound III and CHD-arginine have the same composition and melting point (see above). Slight racemization of Compound III may account for its lower specific rotation. The infrared spectra of Compound III and CHD-arginine are identical, as shown in Fig. 8.

Treatment of Proteins with 1,2-Cyclohexanedione—In order to avoid the formation of multiple products proteins were usually treated in 0.2 N NaOH. Treatment of the ε chain of hemoglobin for 20 hours was reported earlier (1); however, experiments with insulin B chain showed that the condensation reaction is 80, 93, and 100% complete in 4, 14, and 3 hours, respectively. Later experiments were, therefore, conducted with a reaction time of 3 hours. When peptide maps of tryptic digests of untreated (Fig. 9) and CHD-treated (Fig. 10) ε chain of human hemoglobin were compared, the effectiveness of the treatment in blocking tryptic hydrolysis of arginyl bonds was evident. The ε chain has 3 arginyl residues, which are located at the respective COOH termini of tryptic peptides εT4, εT10, and εT14. Blocking of hydrolysis at these positions resulted in the disappearance of tryptic peptides εT4, εT5, εT10, εT11, and εT14 (24, 25), the last being the COOH-terminal tryptic peptide of the ε chain. Five new ninhydrin-positive spots appeared, and these were eluted, hydrolyzed in HCl, and analyzed by high voltage electrophoresis. The qualitative amino acid compositions of these spots are shown in Table I. Except for the substitution of CHD-arginine for arginine, the composition of three of these peptides, which are designated A-1, A-2, and A-3 in Fig. 10, corresponded to εT4, εT10, and εT14, respectively. The other two peptides had compositions consistent with εT12 and εT13, respectively, which are in the insoluble “core” of untreated ε chain. Peptide εT12 represents the only possible ambiguity in the identification of the five new spots as the same qualitative composition occurs in each of the combined peptides, εV T11, εT12, εT13, or εT11,12,13. εT12,13 is likely to be present because the lysyl bond between εT12 and εT13 is difficult to hydrolyze with trypsin, and the combined peptide has been isolated in good yield from tryptic digests of ε chain (24, 25). The qualitative compositions of the other four new spots do not occur in any other tryptic peptide or consecutive sequence of two or three tryptic peptides in the ε chain. The amino acid analyses illustrated in Figs. 7 and 11 indicate that amino acids other than arginine are not affected by the treatment of a protein with CHD.

Electrophoretic analyses of HCl hydrolysates of salmine treated with CHD in 0.2, 0.5, and 1.0 N NaOH are compared in Fig. 12 with a hydrolysate of untreated salmine and with the mixture of products which is produced by the reaction of CHD and arginine in 0.05 N NaOH. Several products were produced by the reaction of CHD with the arginyl residues of salmine; however, arginine was virtually eliminated, and treated salmine was resistant to tryptic hydrolysis in the same way that benzil-
FIG. 12. High voltage paper electrophoresis of acid hydrolysates of untreated salmine (1), of salmine treated with 3-fold excess of CHD in 0.2 N NaOH (2), 0.5 N NaOH (3), and 1.0 N NaOH (4), and of the products of the reaction of CHD and arginine in 0.05 N NaOH (6).

treated salmine was resistant (10). The high arginine content of salmine probably introduces steric influences that interfere with the formation of a homogeneous condensation product.

DISCUSSION

The reaction of CHD with arginine in alkaline solution is relatively rapid, and in hydroxide concentration of 0.2 N or greater results in a homogeneous, water-soluble amino acid. In 0.05 N NaOH several products were observed; however, the rate of disappearance of arginine was just as fast in 0.05 N as in 0.2 N NaOH. If the primary objective of a procedure is to modify arginyl residues without regard to obtaining a homogeneous condensation product of arginine, the reaction may be run at lower hydroxide concentrations. The experiments with salmine indicated that, if arginine is eliminated, tryptic hydrolysis is blocked despite heterogeneity of reaction products. Usually it is desirable to obtain a uniform product in order to simplify subsequent analytical procedures, and it is recommended that reaction in 0.2 N NaOH for 3 hours be used in order to block arginyl bonds in structural studies of proteins. Amino acid analyses and peptide maps of CHD-treated proteins indicate that the reaction is specific for arginine. The only peptide spots that disappeared after CHD treatment of α and β chains of hemoglobin were those of tryptic peptides containing or adjacent to arginyl residues. The new peptides that appeared in the map of α chain were those that contained the condensation product or were solubilized from the core for some unexplained reason. The CHD-arginine peptides of CHD-treated β chain were not located on the map of its tryptic digest (1); one of the arginyl peptides of the β chain is in its core, and the other two are in adjacent tryptic peptides (25, 26). Consequently, blocking tryptic digestion at these positions would result in large insoluble products which might not map well. In maps of tryptic digests of the α and β chain, no new spots that might result from the modification of amino acids other than arginine were observed.

The condensation of CHD with the guanido group of arginine results in an imidazolidinone ring, the stability of which is a convenience in carrying out analytical procedures. It would be desirable, on the other hand, to be able to reverse the reaction and restore the susceptibility of the arginyl position to tryptic hydrolysis; however, experiments directed toward this objective have been unsuccessful.

The high degree of specificity of CHD for arginine suggests its use for modifying proteins for purposes other than blocking tryptic action. Arginine-specific reagents were unknown prior to benilil and little, if anything, is known about the role of arginine in protein structure and enzyme function. For studies on the modification of proteins, CHD has a definite advantage over benzil with respect to solubility in water and molecular size of the arginine derivative as well as of the reagent. The requirement for high basicity for the addition reaction, a disadvantage shared by both, may limit the use of these reagents to proteins which are stable at high pH or which can be restored to their native state after exposure to alkaline conditions.

REFERENCES
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