Cyanogen Bromide Treatment of *N*-Acetylmethionyl Residues without Cleavage†

Frederick H. Carpenter* and Stanley M. Shiigi‡

ABSTRACT: Treatment of *N*-acetylmethionine, *N*-acetylmethionyl peptides, and tris(*N*-acetylmethionyl)insulin with cyanogen bromide in 70% formic acid results to a varying extent in the formation of *O*-acetylhomoseryl residues. This side reaction results in the conversion of *N*-acetylmethionine residues to homoserine residues without cleavage of the methionyl peptide bond. With *N*-acetylmethionyl peptides this side reaction takes place to about 10% and complicates, although does not preclude, the use of the *N*-acetylmethionyl residue as an amino-protecting group in peptide synthesis. With free acetylmethionine over 50% is converted to *O*-acetylhomoserine and less than 40% is converted to *N*-acetylhomoserine lactone.

In connection with studies on the selective chemical modification of insulin (Shiigi, 1972), an amino protecting group was desired which would withstand acidic conditions such as those used for the removal of Boc[1] groups as well as those used in the cyclization step of the phenyl isothiocyanate degradation procedure of Edman (Konigsberg and Hill, 1962). Since bovine as well as porcine and human insulin lack methionine, the *N*-acetylmethionyl (Ac-Met) residue proposed by Gross (1964) as a protective group in peptide synthesis appeared attractive. This group is stable to anhydrous trifluoroacetic acid at room temperature, which treatment is used to remove Boc groups from insulin (Levy and Carpenter, 1967) and to bring about cyclization in the Edman degradation of insulin (Africa and Carpenter, 1970), but it can be removed by treatment with CNBr in 70% formic acid.

The stability of insulin to the conditions of the CNBr cleavage was readily demonstrated. Bovine insulin could be recovered in a crystalline and fully active form after several hours treatment at room temperature with CNBr in 70% formic acid (Shiigi, 1972). However, a study of the removal of the Ac-Met residue from model peptides as well as from insulin revealed the occurrence of a side reaction which limits, although does not preclude, the use of the Ac-Met residue as an amino protecting group.

Materials and Methods

Reagents. Formic acid (88.0–90.0%, Baker and Adamson) was diluted with distilled water to make a 70% solution. Cyanogen bromide (Eastman) was weighed by difference in a stoppered flask and made up to the proper concentration in 70% formic acid immediately before use. Dimethylformamide was purified according to Levy and Carpenter (1967). Tetrahydrofuran (Matheson Coleman and Bell) was purified just before use by distillation from $LiAlH_4$ (Fieser and Fieser, 1967). Crystalline bovine zinc insulin (lots OL VOO and 493-088-078-3 of Eli Lilly and Co.) was converted to insulin hydrochloride by the procedure of Carpenter (1958).

*N-Acetyl-*DL*-Methionine p-Nitrophenyl Ester. N*-Acetyl-DL-methionine (11.5 g, 0.06 mol; Nutritional Biochemical Co.) and *p*-nitrophenol (8.4 g, 0.06 mol) were dissolved in 100 ml of dimethoxyethane. The solution was cooled to 0° and dicyclohexylcarbodiimide (13 g, 0.064 mol) was added. The mixture was stirred for 1 hr at 0° and then left at 5° for 24 hr. The precipitate of dicyclohexylurea was collected by filtration and the solvent was removed from the filtrate on a rotary evaporator. The oily residue was crystallized from 50 ml of ethyl acetate to yield 16.5 g (87%), mp 119–120°.

Anal. Calcd for $C_{13}H_{16}N_2O_5S$: C, 49.99; H, 5.16; N, 8.97; S, 10.26. Found: C, 50.26; H, 5.33; N, 8.85; S, 10.52.

*N-Acetyl-*DL*-methionylglycine.* Ac-Met-Gly, mp 109–112° (lit. 112°; Inglis and Edman, 1970), was prepared by saponification of Ac-Met-Gly-OEt, mp 110–112° (lit. 114°; Lawson *et al.*, 1962), which was prepared from *N*-acetyl-DL-methionine (Nutritional Biochemical Corporation) and glycine ethyl ester by the procedure of Lawson *et al.* (1962).

*N-Acetyl-*DL*-methionyl-*L*-phenylalanine.* L-Phenylalanine methyl ester hydrochloride was prepared by the method of Boissonnass *et al.* (1956) to give a product with mp 157–158° (lit. 159–161°; Schwarz *et al.*, 1957). This ester (4.32 g, 0.02 mol), triethylamine (4.0 g, 0.04 mol), and *N*-acetyl-DL-methionine *p*-nitrophenyl ester (6.88 g, 0.022 mol) were dissolved in 40 ml of dimethylformamide. After the reaction had been stirred for 20 hr at room temperature, the triethylamine hydrochloride was removed by filtration and the solvent was removed from the filtrate on a rotary evaporator. The resulting oil was dissolved in ether from which the product crystallized. The Ac-Met-Phe-OMe was recrystallized from aqueous acetone to yield 2.59 g (30%), mp 113–118°. The ester (2.59 g, 0.073 mol) was saponified in 20 ml of 95% ethanol containing 3.2 ml of 2.5 N sodium hydroxide for 1 hr. The mixture was acidified and the solvent removed on a rotary evaporator. The residue was crystallized and recrystallized from aqueous ethanol to give

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received May 23, 1974.* This study was supported in part by Grants AM 00608 and EY 00813 from the National Institutes of Health. Abstracted from a dissertation presented to the University of California, Berkeley, 1972, as partial fulfillment of the requirements for the Ph.D. degree. A preliminary account of this work was presented previously (Carpenter and Shiigi, 1973).

‡ Supported in part by Training Grant GM-31 from the National Institute of General Medical Sciences.

[1] Abbreviations used are those proposed by the IUPAC-IUB Commision (1966): Boc, *tert*-butoxycarbonyl; CNBr, cyanogen bromide.

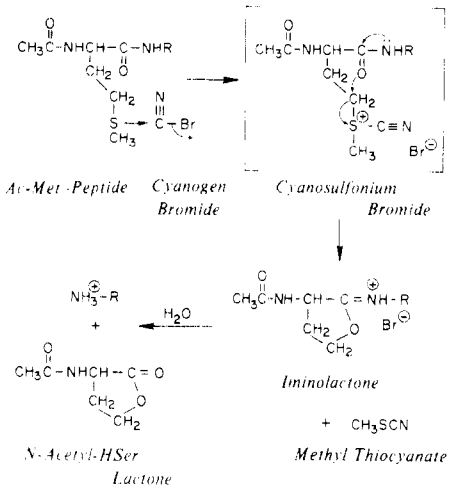

FIGURE 1: Mechanism of CNBr cleavage of *N*-acetylmethionyl peptides, after Gross (1967).

1.96 g (80%) of Ac-Met-Phe with a mp of 153–156°. Amino acid analysis gave a ratio of Met:Phe of 0.99.

Anal. Calcd for $C_{16}H_{22}N_2O_4S$: C, 57.0; H, 6.58; N, 8.32; S, 9.53. Found: C, 56.3; H, 6.34; N, 9.05; S, 9.98.

Tris(N-acetyl- DL-*methionyl)insulin.* The procedure was similar to that used by Levy and Carpenter (1967) to synthesize triaminoacylinsulin derivatives. Insulin hydrochloride (1.2 g, 0.2 mmol) and *N*-acetylmethionine *p*-nitrophenyl ester (0.95 g, 3 mmol) were dissolved in 60 ml of dimethylformamide and triethylamine (0.3 g, 3 mmol) was added. After the solution had been stirred for 20 hr at room temperature, the protein was precipitated by the addition of ether. The precipitate was washed 5 times with acetone, once with ether, and dried *in vacuo*. Excess *p*-nitrophenol was removed from the crude material by passage through a Sephadex G-25 column (3 × 30 cm) at a concentration of 50 mg/ml in the starting buffer (0.05 M Tris, 7 M urea (pH 7.5), and 0.03 M NaCl) used for the DEAE-Sephadex A-25 chromatography. The eluate was placed directly on the DEAE column and eluted with a linear salt gradient from 0.03 to 0.3 M NaCl. The protein peak eluting at 6 mmhos was collected, acidified with acetic acid, desalted by dialysis, and lyophilized.

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl for 6 hr at 120° in evacuated, sealed tubes. After hydrolysis the solvent was removed on a rotary evaporator. Samples containing homoserine lactone were dissolved in 1 ml of 0.2 M pyridinium acetate at pH 7.0 and heated in a sealed tube for 1 hr at 100° (Ambler, 1965). These samples were kept in the pyridine buffer until just before analysis, whereupon they were diluted 1:10 with the citrate sample buffer at pH 2.2. Analyses were performed by the method of Spackman *et al.* (1958) on a Beckman/Spinco amino acid analyzer, Model 120, using the pH 3.30 first buffer and the pH 4.25 second buffer. For methionine determination the buffer change was delayed 30 min in order to elute methionine before the buffer change peak. For hydrolysates of insulin derivatives a seperate analysis for homoserine was performed using a pH 3.1 starting buffer in order to resolve homoserine and glutamic acid. Analyses for *O*-acetylhomoserine were performed on the regular systems using pH 3.30 for the first buffer. *O*-Acetylhomoserine (obtained from Dr. Samuel DiMari of this Department) eluted at 72 min as compared with 60 min for glutamic acid and 82 min for glycine.

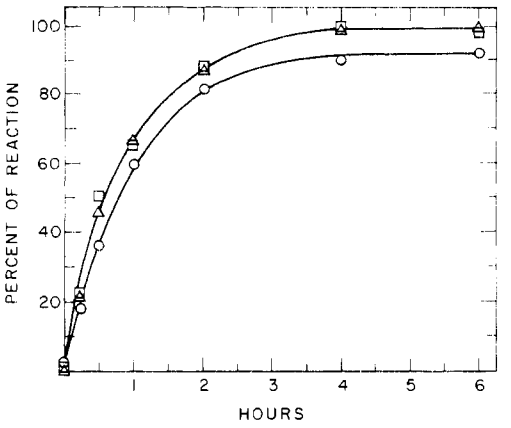

FIGURE 2: Reaction of Ac-Met-Phe (3.6 mM) with CNBr (90 mM) in 70% formic acid at 25°. At various time intervals two aliquots were removed, diluted tenfold with water, frozen, and lyophilized. The residue from one aliquot was dissolved in citrate sample buffer (pH 2.2) and subjected to amino acid analysis for free phenylalanine. The residue from the other aliquots was hydrolyzed in 6 N HCl at 120° for 6 hr. The acid was removed on a rotary evaporator and the residue dissolved in 1 ml of 0.2 M pyridinium acetate buffer at pH 7.0, sealed in an ignition tube, and heated at 120° for 1 hr (Ambler, 1965). Samples were stored in the pyridinium acetate buffer until subjected to analysis whereupon they were diluted 1:10 with the pH 2.2 citrate buffer and immediately placed on the analyzer. The per cent cleavage (○) equals the phenylalanine before/phenylalanine after hydrolysis × 100; the per cent of methionine destroyed (△) equals the methionine/phenylalanine in the acid hydrolysate × 100; the per cent formation of homoserine (□) equals the homoserine/phenylalanine in the acid hydrolysate × 100.

DEAE-Sephadex A-25 Chromatography. Columns (2.5 × 30 cm) were prepared from DEAE-Sephadex A-25 that had been equilibrated with the starting buffer: 0.05 M Tris at pH 7.5, 7 M urea, and 0.03 M NaCl. After addition of the sample (5 mg/ml) in the starting buffer, a linear gradient was applied by running 500 ml of 0.3 M NaCl in the Tris-urea solution into a stirred reservoir containing 500 ml of the starting buffer. Fractions (10 ml) were collected at a flow rate of 50 ml/hr and read for absorption at 277 nm in a Zeiss PMQII spectrophotometer and for conductivity in a Radiometer Type CDM2d meter.

Results

CNBr Splitting of Model Peptides. Figure 1 shows the results expected when a Ac-Met peptide is treated with CNBr (Gross, 1967). Nucleophilic attack by the carbonyl oxygen of the methionyl moiety on the γ-carbon of the cyanosulfonium bromide brings about the release of methyl thiocyanate and the formation of an iminolactone. The latter hydrolyzes to give *N*-acetylhomoserine lactone and the peptide missing the Ac-Met residue (Gross and Witkop, 1961; Gross, 1964, 1967; Inglis and Edman, 1970). The time course of this reaction was followed on several model peptides, including Ac-Met-Phe, with the results shown in Figure 2. At various time intervals, aliquots of the reaction mixture were diluted with water and lyophilized. The residues were subjected to amino acid analysis before and after acid hydrolysis. The hydrolysates were analyzed for homoserine after conversion of homoserine lactone to homoserine just before analysis (Ambler, 1965). The results (Figure 2) indicate virtually 100% destruction of methionine with a concomitant formation of homoserine accompanied by a 90% release of free phenylalanine. Despite the fact that all of the methionine had been converted to homoserine, about 10% of the C-terminal phenylalanine was not released as

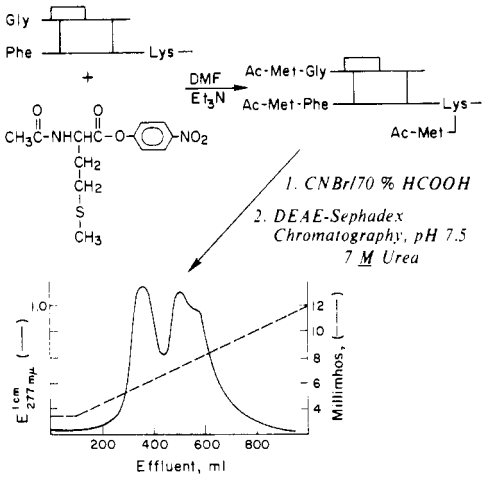

FIGURE 3: Diagram of the synthesis of tris(acetylmethionyl)insulin and its treatment with CNBr. Synthesis of the tris(acetylmethionyl)insulin took place by the treatment of insulin with *N*-acetylmethionine *p*-nitrophenyl ester in dimethylformamide (DMF) and triethylamine (Et_3N) according to the procedure of Levy and Carpenter (1966, 1967). The purified tris(acetylmethionyl)insulin (50 mg/ml) was treated with CNBr (85 mg/ml) in 70% formic acid for 6 hr at 25°. The reaction mixture was diluted tenfold with water and lyophilized. The residue was subjected to DEAE-Sephadex A-25 chromatography (Levy and Carpenter, 1967). Fractions were collected, acidified with acetic acid, dialyzed against water, and lyophilized.

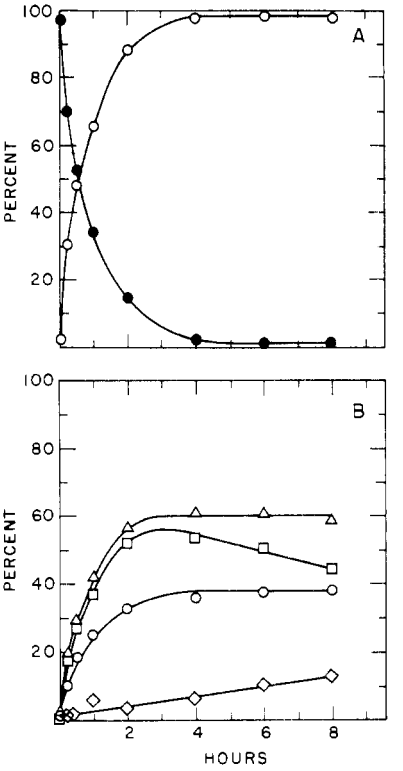

FIGURE 4: Reaction of *N*-acetylmethionine (3.63 mM) with CNBr (90 mM) in 70% formic acid at 25°. Two aliquots were removed at various time intervals and treated as described in Figure 2: (A) (○) total homoserine present in an acid hydrolysate; (●) methionine remaining in an acid hydrolysate; (B) (◇) free homoserine determined before acid hydrolysis of the reaction mixture; (□) *O*-acetylhomoserine determined before acid hydrolysis of the reaction mixture; (△) summation of the *O*-acetylhomoserine and free homoserine; (○) *N*-acetylhomoserine lactone calculated from the difference between total homoserine in an acid hydrolysate and the sum of *O*-acetylhomoserine and free homoserine.

the free amino acid. This indicated that part of the methionine was converted to a homoserine derivative which was still attached to the phenylalanine but which could liberate homoserine and phenylalanine upon acid hydrolysis. Similar results were obtained on treatment of Ac-Met-Gly with CNBr (Shiigi, 1972).

*CNBr Treatment of Tris(N-acetyl-*DL*-methionyl)insulin.* Figure 3 illustrates the formation of a tris(acetylmethionyl)insulin from the treatment of insulin with Ac-Met-ONp according to the procedure used by Levy and Carpenter (1966, 1967) for the addition of amino acid residues to insulin. The resulting tris(acetylmethionyl)insulin, in which Ac-Met residues had been added to the N terminal of each chain as well as to the ϵ-amino group of lysine-B29, was treated with CNBr in 70% formic acid and the reaction mixture was subjected to DEAE-Sephadex chromatography (Levy and Carpenter, 1967). The first peak, which comprises about 40% of the reaction mixture, chromatographs at the same position as insulin in this system. Insulin in crystalline form and with the correct amino acid analysis was isolated in an overall yield of 20% from the material in this peak after removal of salts and urea (Shiigi, 1972). The second peak was a mixture of several components. Amino acid analysis of the isolated components in the second peak indicated the presence of about 0.2 residue of methionine and 0.8 residue of homoserine. Thus, a major portion of the materials in the second peak contains homoserine residues covalently bound to insulin. A minor portion contains one or more methionine residues which have not undergone the CNBr cleavage. Of further interest was the observation that treatment with nitrous acid (Levy and Carpenter, 1970) of the product obtained by lyophilization of the acidic CNBr reaction mixture destroyed all of the homoserine. This result indicates that the bound homoserine formed in the CNBr cleavage of tris(acetylmethionyl)insulin contains a free α-amino group. However, similar nitrous acid treatment of the products in the second peak, which were isolated by chromatography at pH 7.5, had no effect on the homoserine content (Shiigi, 1972). This suggests that during the isolation procedure at neutral pH, an O → N acyl migration took place which covered the exposed α-amino group of the bound homoserine.

Treatment of N-Acetylmethionine with CNBr. A clue as to the nature of the side reaction taking place when Ac-Met peptides and tris(acetylmethionyl)insulin were treated with CNBr was obtained by analysis of the products formed in the treatment of free *N*-acetylmethionine (Figure 4). Figure 4A shows the appearance of homoserine and the disappearance of methionine in the hydrolysate of the reaction mixture. Virtually all of the methionine lost can be accounted for as homoserine in the acid hydrolysate. However, investigation of the products of the reaction mixture before acid hydrolysis revealed the presence of both *O*-acetylhomoserine and free homoserine. Quantitation of the amounts of the homoserine and *O*-acetylhomoserine in the reaction mixture was obtained on the amino acid analyzer with the results shown in Figure 4B. By subtracting the sum of the free homoserine and *O*-acetylhomoserine from the total amount of homoserine present in a complete hydrolysate, a value for *N*-acetylhomoserine lactone was obtained. Inspection of Figure 4B reveals that well over 50% of the initial product formed by the action of CNBr on *N*-acetylmethionine is *O*-acetylhomoserine rather than *N*-acetylhomoserine lactone. Further, the *O*-acetylhomoserine found in this reaction gradually hydrolyzes to free homoserine in the acidic medium. An explanation for these results is shown in

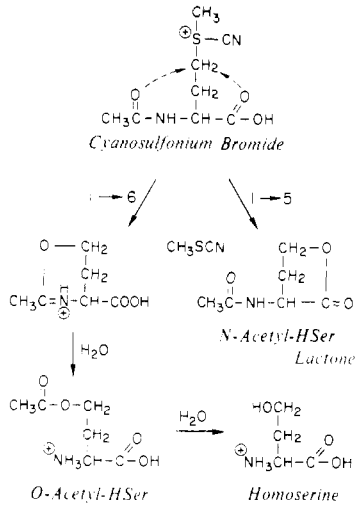

FIGURE 5: Diagram of the reaction pathways during treatment of *N*-acetylmethionine with CNBr in 70% formic acid. The cyanosulfonium bromide eliminates methyl thiocyanate by two pathways: normal attack by the carbonyl oxygen of the methionine moiety to yield a five-membered lactone ring; reverse attack by the carbonyl oxygen of the acetyl moiety to yield a six-membered dihydrooxazine ring. The latter hydrolyzes to give *O*-acetylhomoserine which may in turn hydrolyze to homoserine.

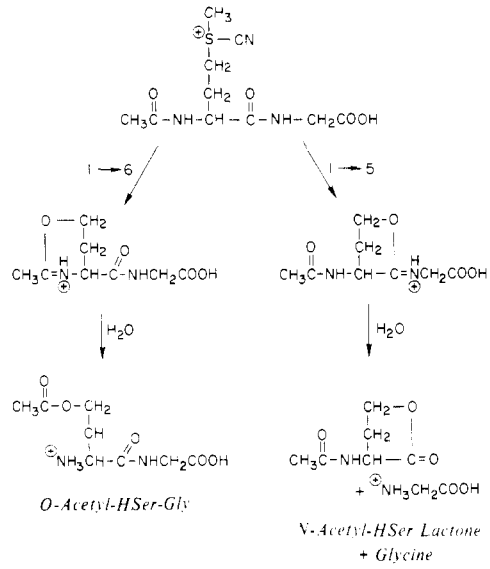

FIGURE 6: Diagram of the reaction pathway in which CNBr cleavage of Ac-Met-Gly yields *N*-acetylhomoserine lactone and glycine as major products and *O*-acetylhomoserylglycine as a minor product.

Figure 5. Attack by the carbonyl oxygen of the acetyl moiety to yield a six-membered dihydrooxazine ring effectively competes with the normal reaction involving one of the oxygens from the carbonyl group of the methionine moiety which yields a lactone with a five-membered ring. Hydrolysis of the six-membered ring yields *O*-acetylhomoserine which in turn gradually hydrolyzes to yield free homoserine.

Demonstration of Reverse Cleavage in Treatment of Ac-Met Peptides with CNBr. The reaction pathway involving the six-membered rather than the five-membered ring is designated here as reverse attack. Figure 6 illustrates the situation that might pertain when an Ac-Met peptide or internal methionine residue in a peptide chain is subjected to CNBr cleavage. The fact that CNBr cleavage of Ac-Met-Phe resulted in a complete conversion of methionine to homoserine with the release of only 90% of the phenylalanine indicates that as much as 10% of the reaction may have followed the route of reverse attack to yield an *O*-acetylhomoseryl residue (Figure 6). That this is really the case is indicated by the results obtained on paper chromatography in acid solvent systems of the reaction mixture of CNBr treatment of Ac-Met-Gly. Figure 7 diagrams the results of such an experiment. Strip A shows the results of chromatography of a glycine standard in 1-butanol–acetic acid–water (4:1:1). Strip B shows the presence of glycine (R_F 0.07) and to a lesser extent another ninhydrin reactive component with an R_F of 0.21 in the CNBr treated peptide. Strip C shows the presence of *N*-acetylhomoserine lactone (R_F 0.66) as visualized by the hydroxylamine–ferric chloride reaction (Waldi, 1965). When the component with the R_F of 0.21 was eluted, hydrolyzed, and subjected to amino acid analysis, glycine and homoserine were present in equal molar amounts (Shiigi, 1972). Thus the substance with R_F 0.21 has the properties (ninhydrin positive, equal molar ratios of homoserine and glycine) expected for the product of reverse attack, *O*-acetylhomoserylglycine. A similar series of experiments performed on Ac-Met-Phe revealed the presence of a substance with the properties (ninhydrin positive, equal molar ratios of homoserine and phenylalanine) expected for O-acetylhomoserylphenylalanine (Shiigi, 1972).

Discussion

The side reaction illustrated in Figure 6 serves as an adequate explanation for the results obtained on CNBr treatment of Ac-Met derivatives. The fact that all of the methionine of Ac-Met-Gly or Ac-Met-Phe is converted to a homoserine derivative without releasing approximately 10% of the C-terminal amino acid residue is accounted for by the reverse attack to yield *O*-acetylhomoseryl residues in which the homoserine is still bound to the C-terminal amino acid. In these cases about 10% of the methionine is converted to homoserine by a mechanism which does not cleave the methionyl peptide bond. The results on tris(acetylmethionyl)insulin have a similar explanation. At least 10% and possibly more of each of the three Ac-Met groups on the insulin undergoes the reverse reaction to yield homoserine residues without splitting the methionyl bond. The fact that these bound homoserine residues are sensitive to nitrous acid treatment indicates that they are present in the acid reaction mixture as *O*-acetyl derivatives. The migration of the acetyl groups from oxygen to nitrogen upon neutralization of the acidic reaction mixture is indicated by the lack of sensitivity of the bound homoserine to nitrous acid treatment in reaction products isolated at pH 7.5. Such an O → N migration is the expected result on neutralization of an *O*-acetylhomoserine derivative (Iwai and Ando, 1967).

The extent of the reverse reaction differs considerably between Ac-Met peptides (about 10%) and free *N*-acetylmethionine (about 50%). This difference may be attributed to the relative nucleophilicity of carbonyl oxygens in the carboxamide residue as compared with the free carboxyl. The carbonyl oxygen of the acetyl moiety in *N*-acetylmethionine is more prone to displace the methyl thiocyanate from the intermediate sulfonium bromide than the carbonyl oxygen of the free carboxyl group despite the formation of the less favored six-membered ring (Figure 5). In the Ac-Met peptides where the carbonyl oxygens of the acetyl and the methionyl moieties are more nearly equal in nucleophilicity, the predominant reaction is in favor of elimination to

give the five-membered lactone ring (Figure 6). In agreement with this explanation is the observation that carbobenzoxymethionylglycine undergoes very little reverse reaction and yields virtually 100% release of glycine when treated with CNBr (Shiigi, 1972). The fact that the carbonyl oxygen of the carbobenzoxy group is less nucleophilic than the carbonyl oxygen of an acetyl group is well known from the relative ease by which these groups form oxazolones during the activation of the carboxyl groups of *N*-carbobenzoxyamino acids as compared with *N*-acetylamino acids (Bodansky and Ondetti, 1966).

The Ac-Met residue occurs in nature as the N-terminal amino acid of several proteins (Steginк *et al.*, 1971). Treatment of these proteins with CNBr in order to cleave off the N-terminal Ac-Met residue can be expected to encounter the same reverse attack experienced with the Ac-Met peptides. Failure to recognize this reaction could complicate the determination of amino acid sequence in that a fraction of N-terminal Ac-Met residues would be converted to homoserine without cleaving the methionyl peptide bond. *N*-Formylmethionine is frequently present as an N-terminal residue in a number of prokaryotic proteins synthesized *in vitro* (Marker *et al.*, 1969), as well as in *Clostridium pasteurianum* rubredoxin (McCarthy and Lovenberg, 1970). Whether or not the formyl group will participate in the reverse reaction has not been established in this work. However, if the relative nucleophilicity argument is valid, one would expect less of the reverse reaction with the formyl group than with the acetyl group.

The CNBr reaction is well established as a specific procedure for splitting peptide chains at internal methionine residues (Gross, 1967). Although this cleavage reaction has proven to be extremely useful in the determinations of primary structure of proteins, the yields of split products rarely approach the theoretical value. In some instances this has been attributed to the masking of methionine residues in the tertiary structure or to the presence of methionine sulfoxide in the peptide chain (Gross, 1967). In other instances, it has been attributed to the presence of -Met-Ser- or -Met-Thr-sequence. In these situations the intermediate iminolactone is attacked by the neighboring hydroxyl group of the serine or threonine to yield homoseryl-*O*-seryl (or threonyl) peptides. Neutralization of the reaction mixture results in an O $\rightarrow$ N migration creating a situation where all of the methionine has been converted to homoserine without cleaving the peptide bond (Shroeder *et al.*, 1969). The present demonstration of the reverse reaction suggests still another explanation for decreased yields of cleavage products. Any fraction of the reaction that proceeds *via* the reverse pathway will yield homoserine without a concomitant amount of peptide bond cleavage. The degree to which the reverse reaction takes place may vary with the nature of the amino acid residues located on the amino side as well as those on the carboxyl side of the methionine residues. In line with the observation reported here on the relative yields of reverse reaction observed for *N*-acetylmethionine as compared with Ac-Met peptides, a C-terminal methionine residue should be more prone to undergo the reverse reaction than an internal methionine residue.

A reverse reaction similar to but not identical with that observed here has been proposed by Awad and Wilcox (1964) to explain the results obtained upon treatment of peptides containing an internal *S*-methylcysteine residue. Reverse reaction through a five-membered iminolactone ring to yield an *O*-acylserine residue takes place in preference to the forward reaction which requires a four-membered iminolactone ring. In the Ac-Met derivative, the reverse reaction through a six-membered ring competes with the forward reaction through a five-membered iminolactone ring. Finally, it should be noted that following the course of the CNBr cleavage by analyzing for the methyl thiocyanate released (Inglis and Edman, 1970) or by analyzing for the amount of homoserine produced (Gross, 1967) may give misleading information on the degree of cleavage of peptide bonds.

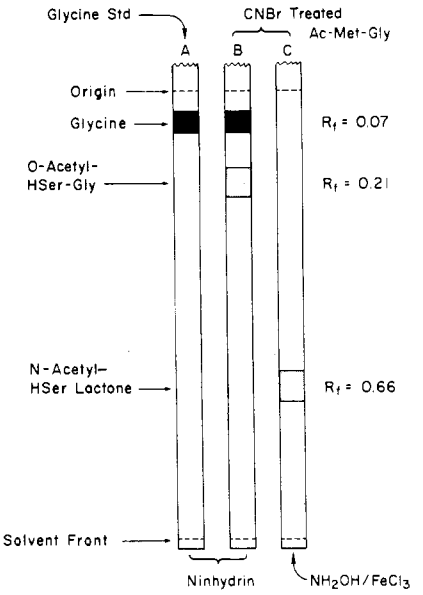

FIGURE 7: Schematic representation of the results of paper chromatography of CNBr-treated Ac-Met-Gly. Descending chromatography on Whatman No. 3-MM paper using 1-butanol-acetic acid-water (4:1:1). Strips A and B were sprayed with 0.2% ninhydrin in acetone to reveal glycine (R_F 0.07) and *O*-acetylhomoserylglycine (*O*-Acetyl-Hser-Gly) (R_F 0.21). Strip C was spyraed with hydroxylamine-ferric chloride (Waldi, 1965) to reveal *N*-acetylhomoserine lactone (*N*-Acetyl-HSer Lactone) (R_F 0.66).

Acknowledgment

We are indebted to Jeannine Caufield Thieme for aid with the amino acid analysis.

References

Africa, B., and Carpenter, F. H. (1970), *Biochemistry 9,* 1962.

Ambler, R. P. (1965), *Biochem. J. 96,* 32 P.

Awad, W., and Wilcox, P. E. (1964), *Biochem. Biophys. Res. Commun. 17,* 709.

Bodansky, M., and Ondetti, M. H. (1966), Peptide Synthesis, New York, N. Y., Interscience, p 137.

Boissonnass, R. A., St. Guttman, P. A., and Jaquenod, J. P. (1956), *Helv. Chim. Acta 39,* 1421.

Carpenter, F. H. (1958), *Arch. Biochem. Biophys. 78,* 539.

Carpenter, F. H., and Shiigi, S. M. (1973), Abstracts, Ninth International Congress of Biochemistry, Stockholm, Sweden, July 1–7, p 71.

Fieser, L. F., and Fieser, M. (1967), Reagents for Organic Synthesis, New York, N. Y., Wiley, p 1140.

Gross, E. (1964), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 23,* 372.

Gross, E. (1967), *Methods Enzymol. 11,* 238.

Gross, E., and Witkop, B. (1961), *J. Amer. Chem. Soc. 83,* 1510.

Inglis, A. S., and Edman, P. (1970), *Anal. Biochem. 37,* 73.
IUPAC-IUB Commission (1966), *J. Biol. Chem. 241,* 2491.
Iwai, K., and Ando, T. (1967), *Methods Enzymol. 11,* 263.
Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237,* 2547.
Lawson, W. B., Gross, E., Folts, C. M., and Witkop, B. (1962), *J. Amer. Chem. Soc. 84,* 1715.
Levy, D., and Carpenter, F. H. (1966), *J. Amer. Chem. Soc. 88,* 3676.
Levy, D., and Carpenter, F. H. (1967), *Biochemistry 6,* 3559.
Levy, D., and Carpenter, F. H. (1970), *Biochemistry 9,* 3215.
Marker, B. A., Clark, B. F. C., and Anderson, J. S. (1969), *Cold Spring Harbor Symp. Quant. Biol. 31,* 279.
McCarthy, K. F., and Lovenberg, W. (1970), *Biochem. Biophys. Res. Commun. 40,* 1053.
Schroeder, W. A., Shelton, J. B., and Shelton, J. R. (1969), *Arch. Biochem. Biophys. 130,* 551.
Schwarz, H., Bumpus, F. M., and Page, I.H. (1957), *J. Amer. Chem. Soc. 79,* 5697.
Shiigi, S. M. (1972), Ph.D. Thesis, University of California, Berkeley, Calif.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Stegink, L. D., Meyer, P. D., and Brummel, M. C. (1971), *J. Biol. Chem. 246,* 3001.
Waldi, D. (1965), *in* Thin Layer Chromatography, Stahl, E., Ed., Academic Press, New York, N. Y., p 483.

Kinetic Studies on the Alkali-Catalyzed Hydrolysis and Epimerization of Model Alkyl and Hydroxyalkyl Di- and Tripeptides†

Burton W. Noll, Carl J. Jarboe,‡ and Louis F. Hass*

ABSTRACT: The present work shows that the alkaline stability of the amide bond in alkyl and hydroxyalkyl dipeptides is a function of the size and the position of the α and α' substituents. When the various alkyl substituents are listed according to their stabilizing effects, the following order is obtained: $(CH_3)_2CHCH_2 \geq (CH_3)_2CH > CH_3CH_2 > CH_3 > H$. The relative alkaline stability of serine- and threonine-containing dipeptides depends upon the position of the R″(HO)CH- group in $NH_2CH(R')CONHCH(R)COO^-$. In the R position, the hydroxyalkyl group facilitates hydrolysis, while in the R′ position, cleavage is impeded. Experimental evidence indicates that peptide bond hydrolysis in dilute alkali at constant ionic strength is strictly a function of a_{OH^-} and is not influenced by potential polyfunctional catalysts (*e.g.,* borate and phosphate). Plots of the pseudo-first-order rate constants (k_{obsd}) *vs.* K_w/a_{H^+} show that solvolysis plays no role in the cleavage of model dipeptides at pH values between 11.5 and 13.6. Based on the above findings, the relative rates of the alkali-catalyzed hydrolysis of each of the amide bonds in the tripeptides, L-Ala-L-Ser-Gly and Gly-L-Ser-L-Ala, can be predicted. In each case, the seryl residue has been found to labilize and to stabilize its own NH_2- and COOH-terminal bonds, respectively. In addition to the studies on hydrolysis, kinetic analyses have been made on alkali-catalyzed epimerization phenomena observed with seryl di- and tripeptides.

During a course of study related to the effect of alkali on the structure of rabbit muscle aldolase, it was found that upon exposure of both the native and the succinyl enzyme to pH 12.5 potassium borate over the temperature range 0–30°, selective peptide bond hydrolysis had occurred yielding a limited number of newly formed NH_2-terminal serine, threonine, and glycine residues (Sine and Hass, 1967, 1969). The invariant nature of the hydrolysis was reflected in the observation that no other amino-terminal residues were liberated after prolonged exposure to alkali. Additional work showed that the phenomenon observed with aldolase was equally applicable to other proteins, such as bovine serum albumin, lysozyme, and ribonuclease A (Hass *et al.,* 1968).

This study has been performed in an effort to gain further insight into the mechanism of selective peptide bond cleavage in proteins exposed to alkali.[1] In addition to investigating the influence of various α-alkyl and α-hydroxyalkyl substituents on the stability of several model di- and tripeptides, we have considered the catalytic effects of various buffers, hydroxide ion, and H_2O on amide bond cleavage. Emphasis has also been placed on investigating the kinetics of serine racemization in seryl peptides. The results derived from the latter studies have provided some understanding of serine's influence on the stability of protein primary structure at high pH values.

Materials and Methods

Materials. Glycylglycine was obtained from Calbiochem. L-Seryl-L-alanine and L-seryl-L-leucine were purchased from Miles Laboratories, Inc. All other dipeptides,

† From the Department of Biological Chemistry, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received May 15, 1974.* This work was supported in part by U. S. Public Health Service Grant HL16647.

‡ Postdoctoral Fellow of the National Institutes of Health.

[1] This report is an expansion of a preliminary communication by Jarboe *et al.* (1971).