peptide, therefore, must be by dissociation of the peptide and rebinding by attachment of its amino-terminal end to the binding sites on the protein (VII). Pepstatin binds tightly (Marciniszyn et al., 1976a) to those regions of the binding site (P_3, P_2, P_1) that had accommodated those residues that became the carboxyl-terminal end of the peptide and can do so as soon as the sites are vacated, even while the peptide is still weakly held to the enzyme at points of contact remote from the catalytic site (Va). This provides an alternative route by which the peptide may dissociate from the protein (V to VIa). In the case of pig pepsinogen activation, that alternative route is apparently faster than the one available in the absence of pepstatin (V to VI). The first chicken peptide liberated is longer than the pig peptide, and apparently its more extensive contact with pseudopepsin holds it more effectively to the distal site(s) on the protein, so that, in the presence of pepstatin, the chicken peptide is released more slowly than is the pig peptide.

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Stepwise Sequence Determination from the Carboxyl Terminus of Peptides†

Joseph L. Meuth,‡ David E. Harris, Francis E. Dwulet,§ Mary L. Crowl-Powers, and Frank R. N. Gurd*

ABSTRACT: The thiocyanate method for stepwise degradation of peptides from their COOH termini [Stark, G. R. (1968) *Biochemistry 7*, 1796] has been investigated. The method involves first the reaction of the COOH-terminal residue with thiocyanate in an activation solvent of acetic acid and acetic anhydride and then cleavage of the COOH-terminal residue as its 2-thiohydantoin by acetohydroxamate in aqueous solution. The two steps of the degradation have been studied by using model peptides, and conditions have been developed for the rapid efficient removal and identification of the COOH-terminal residue of short peptides. The methods have been applied to peptides that have been covalently attached to insoluble supports. In this solid phase version of the degradation, a highly substituted porous glass activated with *N,N'*-carbonyldiimidazole has been prepared for use as the insoluble support. A number of peptides have been coupled to the porous glass, and several rounds of the degradation have been performed on immobilized peptides. High-pressure liquid chromatography provides a rapid, sensitive identification method for the 2-thiohydantoins. In addition, gas-liquid chromatography of the amino acid 2-thiohydantoins and reconversion to the parent amino acid have been used to identify the cleaved residues. The method of sequential degradation has been applied to a number of short model peptides such as Gly-Leu-Tyr, Met-enkephalin, and Val-Leu-Ser-Glu-Gly and has been used to determine the COOH-terminal sequence of 4 residues of a 22-residue cyanogen bromide fragment of pygmy sperm whale myoglobin.

Schlack & Kumpf (1926) proposed a chemical method for the sequential degradation of a peptide from its COOH terminus. The method involved the activation of the COOH-terminal carboxyl group by the formation of a mixed anhydride with acetic acid and then reaction with ammonium thiocyanate to form a peptidyl 2-thiohydantoin. The 2-thiohydantoin was cleaved from the peptide by treatment with base to expose a new COOH-terminal amino acid. Stark (1968) reduced the severity of most of the reaction conditions and used the degradation for subtractive sequencing and then later (Cromwell & Stark, 1969) reported methods for direct identification of the cleaved thiohydantoins by thin-layer chromatography (TLC)[1] or by reconversion to the amino acids (Stark, 1972).

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‡ Present address: Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

§ Present address: Indiana University Medical Center, Clinical Long, Indianapolis, IN 46223.

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Yamashita (1971) described studies on the cleavage of peptidyl thiohydantoins and advocated the use of the acidic form of a cation-exchange resin for mild hydrolysis. The sequence analysis of up to about ten amino acids from the COOH termini of polypeptides has been reported without details (Yamashita & Ishikawa, 1972).

The use of thiocyanic acid rather than thiocyanate salts has resulted in improvements in the coupling step (Kubo et al., 1971). The greater reactivity of thiocyanic acid compared to thiocyanate salts has been shown to facilitate the formation of COOH-terminal 2-thiohydantoins with a number of proteins and protein fragments (Dwulet & Gurd, 1979).

Developments in methods for the identification of amino acid thiohydantoins include gas–liquid chromatography (GLC) (Rangarajan et al., 1973; Dwulet & Gurd, 1977), two-dimensional TLC (Rangarajan & Darbre, 1975), and high-pressure liquid chromatography (HPLC) (Schlesinger et al., 1979; this work).

A number of workers have reported solid-phase versions of the degradation. The attachment of peptides to insoluble supports facilitates the recovery of the peptide after each degradation cycle and shortens the time required for each cycle. Porous glass beads and modified polymers have been used as supports [Williams & Kassell, 1975; Rangarajan & Darbre, 1976; Darbre & Rangarajan, 1975; see also Darbre (1977)].

The present work describes the use of carbonyldiimidazole-activated porous glass as the support for the covalent immobilization of a number of peptides in high yield, the stepwise COOH-terminal sequence analysis of immobilized peptides using thiocyanic acid for the coupling step, and the use of HPLC for the identification and quantitation of the cleaved amino acid 2-thiohydantoins.

Experimental Procedures

Materials

Acetic acid (Mallinckrodt, reagent grade) was redistilled before use. Acetic anhydride (Fisher) was mixed with activated charcoal and redistilled. Acetone (Mallinckrodt, nanograde) was dried over magnesium sulfate. Ammonium thiocyanate (Mallinckrodt, analytical reagent grade) was recrystallized from absolute methanol and stored desiccated. Dimethylformamide (Mallinckrodt, reagent grade) was redistilled from benzene (Stewart & Young, 1969). Triethylamine (Mallinckrodt) was redistilled from ninhydrin before use. All the above reagents were stored at −20 °C. Benzylamine, 2-hydroxy-1-naphthaldehyde, and acetohydroxamic acid (Aldrich) were used without purification. *N*,*N*′-Carbonyldiimidazole (Aldrich) was stored desiccated at −20 °C. (γ-Aminopropyl)triethoxysilane and succinic anhydride were obtained from Pierce. Controlled-pore glass (CPG 10–75 Å, 200–400 mesh) was obtained from Electro-Nucleonics, Inc. The amino acid 2-thiohydantoins were prepared as described (Dwulet & Gurd, 1977). Gly-L-Leu was obtained from Vega. Gly-DL-Leu-DL-Ala, Gly-L-Leu-L-Tyr, and DL-Leu-DL-Phe were obtained from Sigma. Val-Leu-Ser-Glu-Gly was obtained from Cyclo. Met-enkephalin was obtained from Calbiochem-Behring. Crystalline porcine glucagon (lot no. 258-D30-138-2) was provided through the courtesy of Eli Lilly and Co. Major component myoglobin from frozen muscle tissue of pygmy sperm whale (*Kogia breviceps*) supplied by Drs. R. Bonde and K. Beck of the National Fish and Wildlife Laboratory, Gainesville, FL, was prepared by the method of Hapner et al. (1968). The peptides Kb CB-1 and Kb CB-3 were prepared by cyanogen bromide cleavage of pygmy sperm whale myoglobin and were purified by gel filtration by methods essentially identical with those used in the preparation of the cyanogen bromide fragments of the myoglobin of Amazon river dolphin (Dwulet et al., 1975). All other reagents were of the highest available quality. The Altex glass (3 mm × 150 mm) microbore chromatography column and glass column jacket were obtained from Rainin Instrument Co., Inc.

Methods

Preparation of Thiocyanic Acid. The method is based on that of Stokes & Caine (1907) and resembles method III of Dwulet & Gurd (1979).[2]

Preparation of Derivatized Glass. The controlled-pore glass was reacted with (γ-aminopropyl)triethoxysilane by a method based on that described by Robinson et al. (1971). The amino content of the aminoalkylsilyl glass was determined by the method of Schmitt & Walker (1977). The aminoalkylsilyl glass was succinylated by a method based on that of Venter & Dixon (1974).[2] The extent of the succinylation was determined indirectly by quantitating the content of amino groups.

Activation of Succinyl Glass with Carbonyldiimidazole. The activation of the carboxyl groups was performed by a reaction suggested by Staab (1962). One gram of succinyl glass (having between 0.3 and 0.6 mmol of carboxyl group/g of glass) was suspended in 6 mL of anhydrous dimethylformamide, and to the suspension was added solid *N*,*N*′-carbonyldiimidazole to a 20–25-fold molar excess over carboxyl groups. The suspension was degassed and was shaken at room temperature for 20 h. The glass was washed with dimethylformamide and with dichloromethane before being dried under vacuum. The activated glass was stable for at least 18 months when stored desiccated at −20 °C.

Studies on COOH-Terminal Thiohydantoin Formation. The effect of the composition of the coupling solution on the extent of peptidyl 2-thiohydantoin formation was examined by dissolving 1 μmol of Gly-L-Leu in 1.2 mL of a coupling solution. The reaction mixture was stirred at 55 °C for 90 min. At the end of this time, 50-μL samples were removed, dried, and hydrolyzed for amino acid analysis. The decrease in the Leu content of the peptide [determined by amino acid analysis after hydrolysis of the peptide in the absence of 2-mercaptoethanol as described by Dwulet & Gurd (1977)] was taken as a measure of the extent of thiohydantoin formation.

Preparation of Acetyl-Phe-Leu-2-thiohydantoin Using Ammonium Thiocyanate. The compound acetyl-Phe-Leu-2-thiohydantoin used in studies on thiohydantoin cleavage was prepared by a method based on the procedure of Yamashita (1971).[2]

Studies on COOH-Terminal Thiohydantoin Cleavage. The rate of thiohydantoin cleavage was determined spectroscopically. To 3 mL of cleaving reagent in a quartz spectrophotometer cell (1-cm path length) was added 60 μL of acetyl-Phe-Leu-2-thiohydantoin solution (containing 165 nmol of peptide). After the solution was mixed, the decrease in absorbance at 290 nm of the solution was measured with a Cary 14 spectrophotometer.

[1] Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HPLC, high-pressure liquid chromatography; Abu, α-aminobutyric acid; Nle, norleucine; CM-Cys, carboxymethylcysteine; Kb CB-1, NH_2-terminal cyanogen bromide fragment of the major component myoglobin of pygmy sperm whale (*Kogia breviceps*) representing residues 1–55; Kb CB-3, COOH-terminal cyanogen bromide fragment of the major component myoglobin of pygmy sperm whale representing residues 132–153.

[2] See paragraph at end of paper regarding supplementary material.

Table I: COOH-Terminal Solid-Phase Degradation

step	soln	volume (mL)		time (min)
1	coupling soln[a]	system volume (~2 mL) + 1 mL[b]	recycled[c]	90
2	water wash	4	single pass	17
3	cleaving soln[d]	4	single pass	17
4	water wash	4	single pass	17
5	activation soln[e]	4	single pass	17
6	coupling soln	system volume	single pass	9

[a] 1.5 M HSCN in acetone-activation solution (1:4 v/v). [b] See text. [c] Pumping the coupling solution (23 mL) through the column in a single pass gave no improvement in either the efficiency of removal of the carboxyl-terminal residue or the recovery of the cleaved residue. [d] 0.2 M acetohydroxamate in 0.5 M sodium phosphate buffer, pH 8.3. [e] Acetic anhydride-acetic acid (4:1 v/v).

COOH-Terminal Solution Degradation. In a typical experiment, 800 nmol of trifluoroacetic acid treated Gly-L-Leu-L-Tyr was dried in a screw cap tube. The peptide was redissolved in 1.2 mL of acetic acid–acetic anhydride (1:2 v/v), and the solution was stirred in a water bath at 55 °C for 5 min. A 0.21-mL sample of 1.4 M thiocyanic acid in acetone was added (300 μmol of HSCN), and the reaction was stirred at 55 °C for 90 min. The solution was dried under a nitrogen stream and then under vacuum. To the dried peptide was added 1 mL of cleaving solution (Table I), the tube was flushed with nitrogen, and the solution was stirred at 55 °C for 30 min. The solution was allowed to cool to room temperature and was extracted 3 times with 1.2 mL of ethyl acetate. The extracts were combined and were dried under a nitrogen stream. The thiohydantoin was identified and quantitated by HPLC and by amino acid analysis after reconversion to the parent amino acid (Dwulet & Gurd, 1977).

Covalent Attachment of Peptide to Activated Glass. In a typical experiment, 800 nmol of Gly-L-Leu-L-Tyr was dissolved in 1 mL of anhydrous trifluoroacetic acid. The solution was left at room temperature for 5 min, and the trifluoroacetic acid was then removed by a stream of nitrogen. The dried peptide was washed twice with 1 mL of dichloromethane which was removed by a stream of nitrogen and finally dried under vacuum. The peptide was redissolved in 1 mL of dimethylformamide, and 60 μL of triethylamine was added. The solution was added to 100 mg of activated glass in a screw cap tube, and the suspension was degassed. The tube was flushed with nitrogen, and the reaction was shaken at room temperature for 20 h. At the end of this time, the glass was recovered by filtration and was washed with dimethylformamide, water, saturated Na_2HPO_4, water, 1 M acetic acid, water, and finally with acetone. The peptide–glass was dried under vacuum, and the amount of peptide attached was determined by acid hydrolysis and the amino acid analysis of 10–20 mg.

In a number of experiments, after the 20-h peptide attachment the excess activated carboxyl groups were blocked by the addition of 0.1 mL of ethanolamine/100 mg of activated glass. The reaction was shaken at room temperature for 2 h, and the coupled glass was recovered and washed as described above.

COOH-Terminal Solid-Phase Degradation. The degradation was performed with the peptide–glass packed in a 3-mm diameter Altex microbore column modified for the use of plunger ends. The column was fitted with a water jacket and was maintained at 60 °C throughout the degradation. The column was flowed upward at 14 mL/h by using a Sage Instruments Model 375 A tubing pump. The peptide–glass was suspended in activation solution (Table I) for packing in the column (40 mg of peptide–glass occupied approximately 1 cm of the column). The column was washed with 2 mL of coupling reagent (approximately one column volume), and the degradation was then performed as shown in Table I. At the end of step 6, the column was set up to recycle the coupling solution, and the degradative cycle (steps 1–6) was then repeated. The coupling solution was pumped from a reservoir (kept in an ice bath) containing 1 mL of coupling solution, and the column effluent was returned to the reservoir. All other solutions were flowed through the column in a single pass. All solutions were degassed before use.

The column effluent was collected in 1-mL fractions which were analyzed as described below, after changing to the cleaving solution (step 3).

Identification and Quantitation of Amino Acid 2-Thiohydantoins. The GLC of amino acid thiohydantoins and reconversions to the amino acids were performed as described (Dwulet & Gurd, 1977). The HPLC analyses were performed with a Varian Model 5000 chromatograph fitted with a 4-mm diameter × 30 cm MicroPak MCH-10 analytical column and a Varian Universal guard column filled with Vydac reverse-phase packing. The column effluent was passed through a Varichrom variable wavelength detector, and thiohydantoins were detected by their absorbance at 265 nm. The solvents used for the analyses were prepared by dilution of a stock solution of 40 mM sodium acetate buffer, pH 3.7; 2.72 g of sodium acetate trihydrate was dissolved in 400 mL of glass-distilled water, and the pH of the solution was lowered to 3.7 by the addition of glacial acetic acid. The solution was made up to 500 mL with glass-distilled water and was then filtered through a MF-Millipore 0.45-μm filter (Millipore Corp). Solvent A was prepared by diluting the stock buffer solution with 3 volumes of glass-distilled water, and solvent B was prepared by mixing stock buffer solution (100 mL), glass-distilled water (60 mL), and acetonitrile (240 mL).

The column was flowed with solvent A at room temperature at 3 mL/min (typical operating pressure was 180 atm), and the amino acid thiohydantoins were eluted by a gradient program consisting of 0% solvent B for 5 min after injection and then a 30-min linear gradient to 100% solvent B.

Standard solutions of 2-thiohydantoins were made up in methanol. The exact concentrations of the thiohydantoins were determined spectroscopically by dilution of the stock solutions with methanol. The extinction coefficient at 262 nm for all samples except tryptophan and dehydrothreonine was taken to be 17 500 (Cromwell & Stark, 1969).

The cleaving solutions that were analyzed by HPLC were injected directly onto the column. For analysis by GLC or by reconversion, the solutions were extracted 3 times with 1.5 volumes of ethyl acetate. The ethyl acetate extracts were combined and dried by a stream of nitrogen. The dried residues were analyzed as described (Dwulet & Gurd, 1977).

Results

HPLC of Amino Acid 2-Thiohydantoins. The separation of amino acid 2-thiohydantoins by reverse-phase HPLC using gradient elution is shown in Figure 1 and Table II. Most of the 2-thiohydantoins are well resolved. The thiohydantoins that are poorly separated (dehydrothreonine and valine, lysine and methionine) are resolved by isocratic elution (Table III). HPLC provides a rapid and sensitive method for the identification and quantitation of amino acid 2-thiohydantoins.

Thiohydantoin Formation in Solution. The extent of thiohydantoin formation was studied with the model peptide Gly-L-Leu. Amino acid 2-thiohydantoins are stable to acid hydrolysis at 110 °C in the absence of reducing agents

Table II: HPLC of Amino Acid 2-Thiohydantoins[a]

amino acid 2-thiohydantoin	elution time (min)[b]	amino acid 2-thiohydantoin	elution time (min)[b]
Asp	1.36 (8)	Val	9.9 (12)
Gly	1.64 (8)	Lys	11.0 (13)
Gln	2.18 (5)	Met	11.6 (12)
CM-Cys	2.35 (8)	Tyr	11.8 (9)
Glu	2.75 (8)	Ile	13.9 (10)
Ala	3.15 (7)	Leu	14.8 (11)
His	3.32 (4)	Nle	15.5 (10)
Abu	6.3 (3)	Phe	16.2 (9)
Arg	8.8 (3)	Trp	24.8 (9)
Thr	9.7 (11)		

[a] Over the course of approximately 9 months, the elution times of the amino acid 2-thiohydantoins were observed to shorten (by as much as 0.5 min for those amino acid 2-thiohydantoins with elution times greater than 10 min). Reliable identification by HPLC was achieved by the routine use of standards. [b] The number of determinations is given in parentheses.

Table III: HPLC of Amino Acid Thiohydantoins by Isocratic Elution

amino acid thiohydantoin	elution time (min)	
	gradient elution[a]	isocratic elution[b]
Abu	6.3	2.5
Thr	9.7	3.8
Val	9.9	4.3
Lys	11.0	3.9
Met	11.6	5.3
Tyr	11.8	5.3

[a] See text. [b] The elution was performed at 3 mL/min with 90% solvent A.

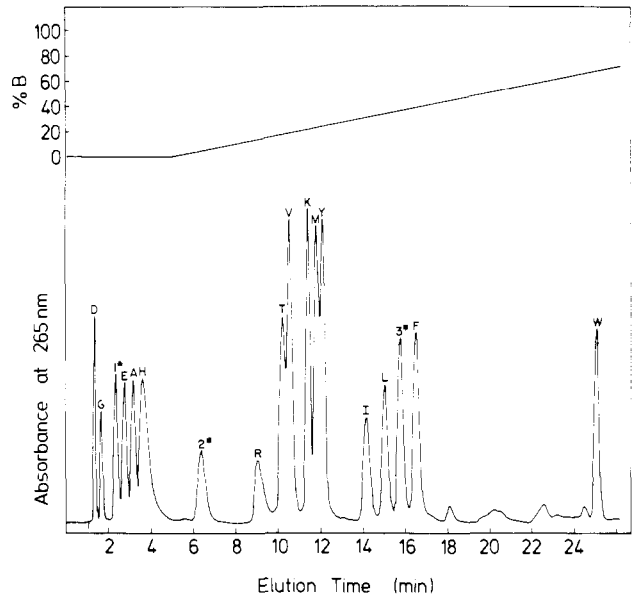

FIGURE 1: Separation of amino acid 2-thiohydantoins by reverse-phase HPLC on a 4-mm diameter × 30 cm MicroPak MCH-10 column as described in the text. The sample size was 0.5–1.0 nmol of each 2-thiohydantoin. The amino acid 2-thiohydantoins are identified by a single letter representing the parent amino acid: (1) CM-Cys; (2) Abu; (3) Nle.

(Dwulet, 1976), and so the decrease in leucine content of the peptide by amino acid analysis was taken as a measure of thiohydantoin formation. The extent of thiohydantoin formation by a number of coupling solutions at 55 °C is shown in Table IV. The presence of the trifluoroacetic acid or of

Table IV: Effect of the Coupling Solution on Thiohydantoin Formation

coupling soln[a]	% thiohydantoin formation[b]
acetic acid–acetic anhydride–trifluoroacetic acid anhydride (33:50:17 v/v)	76
acetic acid–acetic anhydride (1:1 v/v)	72
acetic acid–acetic anhydride (1:2 v/v)	87
acetic acid–acetic anhydride (1:3 v/v)	100
acetic acid–acetic anhydride (1:4 v/v)	100
acetic acid–acetic anhydride (1:9 v/v)	100
trifluoroacetic acid–acetic anhydride (1:9 v/v)	57

[a] The coupling solutions contained in a total volume of 1.2 mL 1 μmol of Gly-L-Leu and 300 μmol of thiocyanic acid. [b] The extent of Leu-2-thiohydantoin formation in the peptide Gly-L-Leu after 90 min at 55 °C was determined as described in the text.

Table V: Rate of Cleavage of Acetyl-Phe-Leu-2-thiohydantoin at Room Temperature

cleavage reagent	half-time of Leu-2-thiohydantoin release
12 M HCl	>30 min
5.7 M HCl	>30 min
saturated triethylamine	42 s
50 mM acetohydroxamate, pH 8.0[a]	90 s
100 mM acetohydroxamate, pH 8.0[a]	40 s
200 mM acetohydroxamate, pH 8.0[a]	30 s

[a] Solutions of acetohydroxamic acid in water were adjusted to pH 8.0 with 5 M NaOH.

trifluoroacetic acid anhydride reduces the coupling efficiency. Trifluoroacetic acid [used in thiohydantoin formation by Kubo et al. (1971)] and trifluoroacetic acid anhydride were used to improve the solubility of proteins and large peptides in the coupling solution at 38 °C (Dwulet & Gurd, 1979). During the course of this work, it was observed that several small to moderate sized peptides were soluble at 55 °C in activating solutions composed of only acetic acid and acetic anhydride. The addition of trifluoroacetic acid or trifluoroacetic anhydride was therefore discontinued, and activating and coupling solutions having the composition acetic anhydride–acetic acid (4:1 v/v) were used for all subsequent solution and solid-phase studies. It should be noted that the solubility of a large peptide in the coupling solution should be less of a problem for the solid-phase degradation since the peptide would be distributed as a uniform film on the solid support and its covalent immobilization should minimize aggregation.

Thiohydantoin Cleavage in Solution. The rate of cleavage of acetyl-Phe-Leu-2-thiohydantoin by a number of cleavage solutions is shown in Table V. The slow cleavage with 6 and 12 M HCl was similarly observed by Dwulet (1976), and Stark (1968) reported a half-time of 26 min for the cleavage by 10 M HCl of the peptidyl thiohydantoin derived from the oxidized insulin B chain. In contrast, the deacetylation of 1-acetyl-2-thiohydantoins by concentrated HCl occurs with half-times of about 2 min (Stark, 1968; Darbre, 1977). The reason for this observed difference in thiohydantoin cleavage is not known.

The cleavage of acetyl-Phe-Leu-2-thiohydantoin by saturated triethylamine (Dwulet & Gurd, 1979) is rapid (Table V). Preliminary studies on the use of this volatile cleavage reagent in COOH-terminal degradations of short peptides in solution are described below. The cleavage with acetohydroxamate [originally used by Stark (1968)] is rapid and occurs under mild conditions. A cleavage solution of 0.2 M acetohydroxamate in sodium phosphate buffer, pH 8.3, has

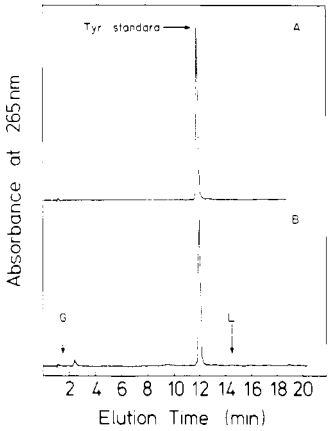

FIGURE 2: Identification by HPLC of the amino acid 2-thiohydantoin formed by a single round of the degradation performed in solution on the peptide Gly-L-Leu-L-Tyr. HPLC of (A) Tyr-2-thiohydantoin standard (22 nmol) and (B) ethyl acetate extract of the cleavage solution from the degradation of 800 nmol of Gly-L-Leu-L-Tyr redissolved in 400 μL of methanol. (2.5% of the sample was injected for analysis.)

been used in solution studies and in solid-phase versions of the degradation (see below).

COOH-Terminal Solution Degradation. The peptides Gly-L-Leu, DL-Leu-DL-Phe, and Gly-L-Leu-L-Tyr were each subjected to one round of the COOH-terminal solution degradation in order to determine the yield of thiohydantoin. From the degradation of Gly-L-Leu, 100% of the expected Leu-2-thiohydantoin was recovered (by GLC), from the degradation of DL-Leu-DL-Phe, 94% of the expected Phe-2-thiohydantoin was obtained (by GLC), and from the degradation of Gly-L-Leu-L-Tyr, 108% of the expected Tyr-2-thiohydantoin was recovered (by HPLC, see Figure 2). In the degradation of Gly-L-Leu-L-Tyr, no significant preview (which would have been indicated by the presence of Gly-2-thiohydantoin or Leu-2-thiohydantoin in the cleavage solution) was observed.

The method provides a rapid procedure for determining the COOH-terminal residue of a peptide. It is limited to those peptides that are soluble in the coupling solution and may be conveniently used for only a single cycle since removal of the cleaving reagents from the peptide (achieved by gel filtration for large peptides; Stark, 1968) is likely to be a time-consuming procedure.

The combination of the thiocyanic acid coupling step with a cleavage performed with saturated triethylamine constitutes a method for stepwise degradation using volatile reagents. Preliminary studies on such a procedure have been performed by using model peptides.[2]

Attachment of Peptides to Porous Glass. It has been argued that the most useful supports for solid-phase NH_2-terminal sequencing of proteins have been prepared by the derivatization of glass with aminoalkylsilanes (Laursen & Machleidt, 1980). These supports have also been used for the covalent immobilization of peptides for COOH-terminal sequencing (Williams & Kassell, 1975; Rangarajan & Darbre, 1976). The methods here describe the preparation of a highly substituted aminopropylsilyl glass and the modification and activation of this glass for peptide attachment. The high efficiency of peptide attachment [Table VI; compare Williams & Kassell (1975)] results from a number of factors: the high degree of substitution of the glass (typically 0.3–0.6 mmol of reactive group/g of glass), the use of carbonyldiimidazole to give a highly activated glass, the use of anhydrous conditions for coupling peptides to the glass, and the treatment of peptides

Table VI: Attachment of Peptides to Carbonyldiimidazole-Activated Porous Glass

peptide	amount of peptide	amount of activated glass (mg)[a]	attachment (%)
Gly-Leu	1.0 μmol	100	>95
Gly-Leu-Ala	1.0 μmol	100	>95
Gly-Leu-Tyr	0.8 μmol	200	93
Met-enkephalin	1.0 μmol	100	57
Met-enkephalin	0.75 μmol	200	85
Kb CB-3[b]	0.35 μmol	150	>95
Kb CB-3[b]	0.24 μmol	150	67
glucagon	90 nmol	150	76
Kb CB-1[c]	0.18 μmol	100	29

[a] All couplings were performed in 1 mL of dimethylformamide. [b] COOH-terminal CNBr peptide from pygmy sperm whale myoglobin consisting of residues 132–153. [c] NH_2-terminal CNBr peptide from pygmy sperm whale myoglobin consisting of residues 1–55.

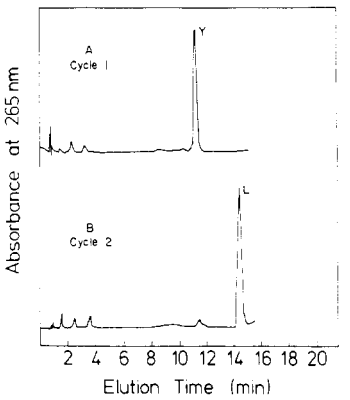

FIGURE 3: Identification by HPLC of the amino acid 2-thiohydantoins formed after sequential degradation of glass-bound Gly-L-Leu-L-Tyr (660 nmol of peptide attached to 175 mg of glass). The cleavage solution of each cycle (4 mL) was collected in 1-mL fractions which were analyzed by HPLC. HPLC of (A) the second 1-mL fraction of the cleavage solution of cycle 1 (the peak eluting at 11.3 min was identified by comparison with standards as 1.74 nmol of Tyr-2-thiohydantoin) and (B) the first 1-mL fraction of the cleavage solution of cycle 2 (the peak eluting at 14.3 min was identified as 2.08 nmol of Leu-2-thiohydantoin). 1% of each sample was injected for analysis.

with trifluoroacetic acid to improve their solubilities in the coupling solvent. The improvement in solubility of a peptide in an organic solvent such as dimethylformamide after trifluoroacetic acid treatment has been previously reported (Tarr, 1977; Laursen & Machleidt, 1980).

COOH-Terminal Solid-Phase Degradation. The results from experiments with short peptides are shown in Table VII and Figure 3. Amino acid analysis of the peptide–glass after sequencing indicates that the degradation generally results in the removal of approximately 90% of the carboxyl-terminal residue. This compares with typical values of 94–98% for the removal of the amino-terminal residue by the Edman degradation (Walsh et al., 1981). The recovery yields of the 2-thiohydantoins obtained after each cycle are lower than 90%. The reason for the low recovery of the 2-thiohydantoins observed in the solid-phase degradation is not known. Solvents and reagents used in a degradative cycle have been analyzed by HPLC and by reconversion and amino acid analysis, and thiohydantoins were detected only in the cleavage reagent.

The result of performing six cycles of the degradation on the peptide Kb CB-3 is shown in Table VIII and Figure 4. Identification of the carboxyl-terminal sequence is possible for the first three residues, and tentative assignment of the fourth residue can be made. The sequence agrees with that determined by conventional techniques (Dwulet et al., 1977; M. L.

Table VII: Sequencing of Peptides Attached to Porous Glass from the Carboxyl Terminus

peptide	amount of peptide (nmol)	amount of porous glass (mg)	no. of degradative cycles performed	amino acid 2-thiohydantoins identified in cleavage soln[a] round 1	round 2	round 3	composition of peptide attached to glass after sequence anal[b]
Gly-Leu	1060	100	1	Leu 45% (GLC)			Gly (1.0), Leu (<0.02)
Gly-Leu-Ala	999	100	2	Ala 40% (GLC)	Leu 46% (GLC)		Gly (1.0), Leu (0.07), Ala (<0.02)
Gly-Leu-Ala	1075	100	2	Ala 63% (HPLC)	Leu 47% (HPLC)		Gly (1.0), Leu (0.05), Ala (<0.02)
Gly-Leu-Tyr	680	176	1	Tyr 75% (HPLC)			Gly (1.0), Leu (0.91), Tyr (0.08)
Gly-Leu-Tyr	659	175	2	Tyr 61% (HPLC)	Leu 39% (HPLC)		Gly (1.0), Leu (0.31), Tyr (0.20)
Met-enkephalin[c]	412	129	2	Met 56% (HPLC)	*d*		Tyr (0.77), Gly (2.0), Phe (0.97), Met (0.11)
Met-enkephalin[c]	508	100	4	Met 45% (GLC)	Phe 63% (GLC)	*e*	Tyr (0.77), Gly (0.85), Phe (0.05), Met (<0.02)
Val-Leu-Ser-Glu-Gly	136	124	4	Gly 75% (HPLC)	Glu 42% (HPLC), Gly 9% (HPLC)	*f*	Val (0.88), Leu (0.62), Ser (0.33), Glu (0.23), Gly (0.17)

[a] Thiohydantoins were identified and quantitated by the indicated techniques (the percent recovery of each thiohydantoin is shown). They were in addition reconverted to the amino acid and identified by amino acid analysis. [b] An amino acid composition was calculated relative to that of the peptide attached to glass before the sequence analysis. This was done to correct for the low recoveries observed for the NH_2-terminal residues that were covalently linked to the glass. [c] Tyr-Gly-Gly-Phe-Met. [d] The degradation was performed omitting the activation prior to the second cycle (step 5, Table I). No amino acid thiohydantoin was observed in the second cycle. [e] Low yields of Gly-2-thiohydantoin were obtained in the third and fourth rounds (<10% of those expected). The low yields were partly a result of the low recovery of Gly-2-thiohydantoin observed in the ethyl acetate extraction of the cleaving solution. [f] No amino acid 2-thiohydantoins were detected in the third and fourth rounds.

Table VIII: Amino Acid Composition[a] of Peptide Kb CB-3 before and after COOH-Terminal Sequencing

amino acid	expected from sequence[b]	isolated peptide Kb CB-3	peptide attached to glass: before sequencing	peptide attached to glass: after six rounds of degradation
Asp	1	0.98	1.02	1.00
Thr	1	1.03	0.92	1.05
Glu	3	2.78	2.72	1.95
Gly	2	1.87	2.09	0.60
Ala	3	2.93	2.70	2.81
Ile	1	0.87	0.83	1.00
Leu	3	2.76	2.84	2.37
Tyr	2	1.50	1.43	0.82
Phe	1	1.05	1.09	1.07
Lys	4	3.34	3.27	3.54
Arg	1	1.10	1.06	1.01

[a] Samples were hydrolyzed with 6 M HCl at 110 °C for 24 h and were analyzed on a Beckman 120C amino acid analyzer. Only the values for those amino acids present in the analyses are presented. An amino acid composition was calculated by using a mean value for the amount of analyzed peptide based on assumed integral values for the amino acids Asp, Ala, and Phe. [b] Thr-Lys-Ala-Leu-Glu-Leu-Phe-Arg-Lys-Asp-Ile-Ala-Ala-Lys-Tyr-Lys-Glu-Leu-Gly-Tyr-Gln-Gly (M. L. Crowl-Powers, unpublished results).

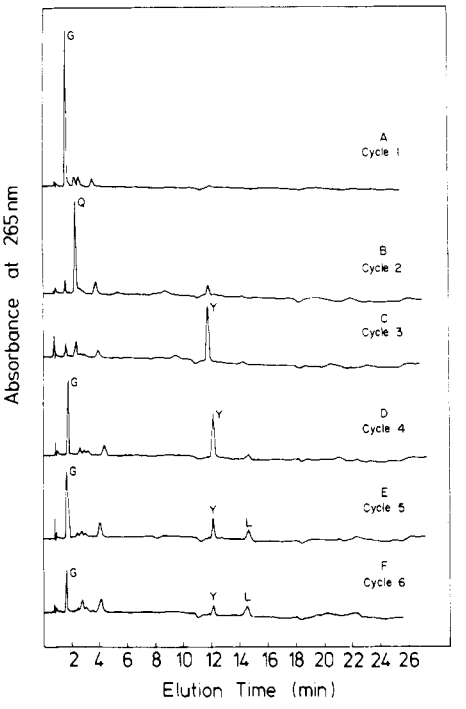

FIGURE 4: Identification by HPLC of the amino acid 2-thiohydantoins formed after sequential degradation of glass-bound Kb CB-3 (350 nmol of peptide attached to 150 mg of glass). HPLC of the first 1-mL fraction of the cleavage solution of (A–F), cycles 1–6, respectively. The major peaks of amino acid 2-thiohydantoins are identified by a single letter representing the parent amino acid (G, Gly; Q, Gln; Y, Tyr; L, Leu). 1% of each sample was injected for analysis.

Crowl-Powers, unpublished results) and is identical with the carboxyl-terminal sequence of sperm whale myoglobin (Edmundson, 1965). Low yields of 2-thiohydantoins were obtained. For example, the carboxyl-terminal glycine was obtained in approximately 30% yield, and the glutamine, tyrosine, and glycine in rounds 2, 3, and 4, respectively, were obtained in 10–15% yield. The reason for the low yields of thiohydantoins obtained in the solid-phase degradation relative to those performed in solution is being explored.

Discussion

The development of methods for the stepwise degradation of a peptide from the carboxyl terminus has been the objective of several studies. Such methods would complement the Edman degradation (Edman, 1950) and would greatly simplify the task of protein sequencing. The thiocyanate degradation of Stark (1968) has been the most widely studied chemical method. A number of improvements in the procedure have decreased the time required for each degradation cycle and have facilitated the identification of the cleaved residue. To overcome problems associated with peptide solubility and with the isolation of the peptide after each degradation cycle, procedures have been developed for performing the degradation on peptides that have been immobilized by covalent attachment through their amino groups to a solid support.

A number of features of the degradation reported here should be noted. These include (a) the use of thiocyanic acid rather than thiocyanate salts for the coupling step [the greater

reactivity of thiocyanic acid over the salts has been discussed (Dwulet & Gurd, 1979)], (b) the use of carbonyldiimidazole to give a highly reactive acylimidazole derivative of the solid support [carbonyldiimidazole has been used for peptide attachment in solid-phase versions of the Edman degradation (Laursen, 1966, 1971), and the high efficiency of the couplings obtained with this reagent has been noted (Laursen & Machleidt, 1980)], (c) the reduction of the time required for each degradation cycle to 3 h which compares with the 5–6 h previously reported (Williams & Kassell, 1975; Darbre, 1977), (d) the use of a column for the reaction vessel which is permitted by the physical properties of the solid support, and (e) the use of HPLC for the identification and quantitation of amino acid 2-thiohydantoins. HPLC is a particularly useful method for the identification of the cleaved amino acid 2-thiohydantoins since it permits the direct analysis of the cleavage solution. Other methods of analysis such as GLC or reconversion require that the amino acid 2-thiohydantoins be extracted from the aqueous cleavage solution. Some amino acid 2-thiohydantoins are extracted in low yield (see footnote *e* to Table VII), and Glu-2-thiohydantoin (obtained in the second round of degradation of Val-Leu-Ser-Glu-Gly, Table VII) was extracted only after acidification of the cleavage solution.

The results presented here indicate that short peptides may be degraded with high efficiency in a stepwise manner from the carboxyl terminus. The typical efficiency of 90% removal of the carboxyl-terminal residue implies that about ten rounds of the degradation might be performed before the identification of the cleaved residue would become difficult. Difficulties in residue identification arising from a combination of a low yield of the cleaved residue and a large carry-over appear to result from failures of the coupling steps to go to completion. Such difficulties have been observed in degradations performed with some batches of thiocyanic acid that had been stored for longer than 2 weeks at −20 °C. Reagent batches may be tested by performing a single degradative round in solution on the peptide Gly-Leu-Tyr. In those cases where the yield of cleaved Tyr-2-thiohydantoin is less than 90%, the reagents are discarded.

The lower than expected recoveries of the cleaved 2-thiohydantoins (Table VII) may be a result either of decomposition of the 2-thiohydantoins or of adsorption of the cleaved 2-thiohydantoins by the porous glass support. 2-Thiohydantoins are unstable in aqueous solution at high pH and at high temperatures and decompose by a number of reactions including hydrolysis to thiohydantoic acids (Scoffone & Turco, 1956) or by oxidation by molecular oxygen. The stability of thiohydantoins has been discussed by Stark (1968) and by Cromwell & Stark (1969). Studies on the stabilities of the 2-thiohydantoins of Tyr and Leu in the present cleaving solution have shown that they exhibit good stabilities at room temperature but that they decompose at 55 °C with half-times of disappearance of about 100 min (results not shown). In the presence of porous glass, the rates of disappearance at 55 °C are increased. If the increased rates of disappearance of amino acid 2-thiohydantoins in the presence of porous glass are due to adsorption, the 2-thiohydantoins are being bound essentially irreversibly. They are not removed when the porous glass is washed with a variety of aqueous and nonaqueous solvents, and no amino acids are recovered when the porous glass is heated with 6 M HCl under conditions that would be expected to convert 2-thiohydantoins into their parent amino acids. It is thus likely that the increase in the rate of disappearance of an amino acid 2-thiohydantoin in the presence of porous glass results from an increase in the rate of decomposition and not by adsorption onto the porous glass. The low recoveries of the cleaved 2-thiohydantoins are generally not a problem for residue identification since the methods of thiohydantoin identification (HPLC and GLC) are capable of detecting very small amounts.

It was anticipated by Stark (1968) that proline and aspartic acid would not be degraded when they occurred at the carboxyl terminus of a peptide. In the degradation of ribonuclease A reported by Yamashita (1971), aspartic acid (the fourth amino acid residue from the carboxyl terminus) was recovered in low yield, indicating that thiohydantoin formation can occur with this amino acid. Kubo et al. (1971) reported the preparation of prolinethiohydantoin and presented evidence that thiohydantoin formation with proline occurs by deacylation (cleavage of the carboxyl-terminal prolinethiohydantoin occurs during its formation). In the solid-phase degradation of peptides containing proline, it would thus be necessary to collect and analyze both the coupling solution and the cleavage solution of each round.

A number of alternative stepwise methods for the chemical degradation of peptides have been proposed [see Darbre (1977) and Laursen & Machleidt (1980) for descriptions], but none have reached the degree of development of the thiocyanate degradation. As an alternative to chemical methods of sequencing, the release of amino acids from a peptide by carboxypeptidase digestion (Ambler, 1967a,b) has been widely used for determining the carboxyl-terminal sequence. Problems with the enzymatic approach mainly result from the fact that the released amino acids do not necessarily make their appearance in a stepwise manner. Because of the specificities of the available carboxypeptidases, misinterpretation of the necessary kinetic experiments is possible (Ambler, 1967a).

The solid-phase thiocyanate degradation provides a rapid method for the determination of a short sequence at the carboxyl terminus of a peptide. The present method has been applied to a number of model peptides and has been used to determine the sequence of four residues at the COOH terminus of a 22-residue protein fragment by using 350 nmol of the peptide. Results are presented showing the removal of alanine, glycine, glutamic acid, glutamine, leucine, methionine, phenylalanine, and tyrosine from the carboxyl-terminal position of peptides and their identification as amino acid 2-thiohydantoins. In addition, the results obtained in the degradation of Kb CB-3 (Figure 4 and Table VIII) indicate that the degradation can be performed on peptides containing most of the amino acids commonly found in proteins. The method should have some application in the identification of overlapping sequences, the establishment of the nature and number of COOH-terminal groups in a preparation, and the confirmation of the purity of a peptide preparation. The extension of this work to longer sequences is being undertaken.

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Supplementary Material Available

A description of the preparation of thiocyanic acid and of aminoalkylsilyl glass, the succinylation of aminoalkyl glass,

the preparation of acetyl-Phe-Leu-2-thiohydantoin, and preliminary studies on a stepwise solution phase carboxyl-terminal degradation (4 pages). Ordering information is given on any current masthead page.

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Kinetic Mechanism of the Reaction Catalyzed by Dihydrofolate Reductase from *Escherichia coli*†

Stuart R. Stone and John F. Morrison*

ABSTRACT: The kinetic mechanism of the reaction catalyzed by dihydrofolate reductase from *Escherichia coli* has been investigated by using progress curve, initial velocity, product inhibition, and dead-end inhibition studies as well as isotope effects. The results indicate that the reaction conforms to a random mechanism involving two dead-end complexes, viz., enzyme–DHF–THF and enzyme–NADP–DHF. At higher concentrations, DHF causes substrate inhibition by combining at the NADPH binding site on the enzyme. The steady-state velocity data can be analyzed adequately on the basis that rapid-equilibrium conditions apply. However, this can be only an approximate description of the reaction since the isotope effects observed with NADPD demonstrate clearly that catalysis cannot be rate limiting at pH 7.4. The choice of conditions for analysis of progress-curve data is discussed in the Appendix.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF)[1] to 5,6,7,8-tetrahydrofolate (THF). THF is an essential cofactor in a number of one-carbon transfer reactions including the biosynthesis of thymidylate. Thus, inhibition of dihydrofolate reductase can lead to a deficiency of thymidylate and to the disruption of DNA biosynthesis. Inhibitors of the enzyme have found clinical applications in the treatment of neoplastic and microbial diseases (Blakley, 1969). Because of its pharmacological importance, dihydrofolate reductase has been studied extensively (Gready, 1981), but despite the many detailed studies on the enzyme, its kinetic mechanism has not been established. It has been proposed that the reaction catalyzed

† From the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra City A.C.T. 2601, Australia. *Received January 5, 1982.*

[1] Abbreviations: DHF, 7,8-dihydrofolate; THF, 5,6,7,8-tetrahydrofolate; Tris, tris(hydroxymethyl)aminomethane; Mes, 4-morpholineethanesulfonic acid.

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