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Carboxy-Terminal Sequencing: Formation and Hydrolysis of C-Terminal Peptidylthiohydantoins†

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ABSTRACT: Proteins and peptides can be sequenced from the carboxy terminus with isothiocyanate reagents to produce amino acid thiohydantoin derivatives. Previous studies in our laboratory indicated that the use of trimethylsilyl isothiocyanate (TMS-ITC) as a coupling reagent significantly improved the yields and reaction conditions and reduced the number of complicating side products [Hawke et al. (1987) *Anal. Biochem. 166*, 298]. The present study further explores the conditions for formation of the peptidylthiohydantoins by TMS-ITC and examines the cleavage of these peptidylthiohydantoin derivatives into a shortened peptide and thiohydantoin amino acid derivative. Schizophrenia-related peptide (Thr-Val-Leu) was used as a model peptide and was treated with acetic anhydride and TMS-ITC at 50 °C for 30 min, and the peptidylthiohydantoin derivatives were isolated by reverse-phase HPLC and characterized by FAB-MS. The purified derivatives were subjected to a variety of cleavage conditions, and rate constants for hydrolysis were determined. Hydrolysis with acetohydroxamate as reported originally by Stark [(1968) *Biochemistry 7*, 1796] was found to give excellent cleavage of the terminal thiohydantoin amino acid, but also led to the formation of stable hydroxamate esters of the shortened peptide which are poorly suited for subsequent rounds of degradation. Hydrolysis with 2% aqueous triethylamine under mild conditions (1-5 min at 50 °C) was found to be more suitable for carboxy-terminal sequence analysis by the thiocyanate method. The shortened peptide, which could be isolated and subjected to a second round of degradation, and the released thiohydantoin amino acid are formed in good yield (90-100%). Several other small peptides containing 15 different C-terminal amino acid side chains were also investigated in order to examine any interfering reactions that might occur when these side chains are encountered in a stepwise degradation using the thiocyanate chemistry. Quantitative yields of peptidylthiohydantoins were obtained for all the amino acids examined with the following exceptions: low yields were obtained for C-terminal Glu or Thr, and no peptidylthiohydantoins were obtained for C-terminal Pro or Asp. Asparagine was found to form cyclic imides (64%) at the penultimate position (C-2) during hydrolysis of the peptidylthiohydantoins by 2% aqueous triethylamine. Cleavage of C-terminal Asn under these conditions led to the formation of the expected shortened peptide (69%), but also to the formation of a shortened peptide (31%) with a C-terminal amide. Problems with Glu and Thr could be solved by minimizing the reaction time with acetic anhydride. Addition of a nucleophile prior to the coupling reaction was used to allow partial degradation of C-terminal Asp. Under a variety of conditions, no peptidylthiohydantoins were formed when Pro was at the C-terminus. Although at this point Pro remains a problem, the combined procedure of reaction with TMS-ITC and hydrolysis with 2% aqueous triethylamine appears suitable for adaptation to a solid-phase extended degradation of peptides and proteins.

The development of methods for the sequential degradation of proteins and peptides from the carboxy terminus is a goal of this laboratory and has been the objective of several studies [for review see Ward (1986) and Rangarajan (1988)]. Such a method would complement existing N-terminal degradations based on the Edman chemistry (Edman, 1950). The most widely studied method and probably the most attractive be-

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cause of its similarity to the Edman degradation has been the conversion of amino acids into thiohydantoins. This reaction, originally observed by Johnson and Nicolet (1911), was first applied to the sequential degradation of proteins from the carboxy terminus by Schlack and Kumpf (1926). These authors reacted ammonium thiocyanate, dissolved in acetic acid and acetic anhydride, with N-benzoylated peptides to form carboxy-terminal 1-acyl-2-thiohydantoins. Exposure to strong base was used to liberate the amino acid thiohydantoin and generate a new carboxy-terminal amino acid. The main disadvantages of this procedure have been the severity of the conditions required for complete derivatization of the C-terminal amino acid and for the subsequent cleavage of the peptidylthiohydantoin derivative into a new shortened peptide and an amino acid thiohydantoin derivative. Since this work was published, numerous groups have tried to reduce the severity of the conditions required, particularly in the cleavage of the peptidylthiohydantoin, in order to apply this chemistry to the sequential degradation of proteins from the carboxy-terminal end. Lesser concentrations of sodium hydroxide (Waley & Watson, 1951; Kjaer & Eriksen, 1952) than originally used by Schlack and Kumpf (1926) and of barium hydroxide (Turner & Schmerzier, 1954) were found to effectively cleave peptidylthiohydantoins. Other groups (Tibbs, 1951; Baptist & Bull, 1953) used acidic conditions based on the original procedure used by Johnson and Nicolet (1911) for the deacetylation of amino acid thiohydantoins. These authors added concentrated hydrochloric acid to the coupling solution to cause cleavage of the peptidylthiohydantoin bond. Unlike hydroxide which was shown to cause breakdown of the thiohydantoin amino acids (Scoffone & Turco, 1956), hydrochloric acid was shown not to destroy the amino acid thiohydantoins (Fox et al., 1955; Stark, 1968). Cromwell and Stark (1969) showed that concentrated hydrochloric acid could be used to cleave the thiohydantoin amino acid at room temperature. The major drawback with this procedure was that when applied to proteins no more than two or three cycles could be performed. Yamashita (1971) found that cleavage of peptidylthiohydantoins could be done in a repetitive manner with a protonated cation-exchange resin. Application of this procedure to 100-μmol quantities of papain and ribonuclease was reported to give 14 and 10 cycles, respectively, although no details were given (Yamashita & Ishikawa, 1971). Stark (1968) reported that certain organic bases such as morpholine or piperidine could be substituted for sodium hydroxide, and along the same lines, Kubo et al. (1971) reported that aqueous triethylamine (0.5 M) could be used to effectively cleave peptidylthiohydantoins. Stark (1968) appeared to have solved the cleavage problem by introducing acetohydroxamic acid in aqueous pyridine at pH 8.2 as a cleavage reagent. This reagent was shown to rapidly and specifically cleave peptidylthiohydantoins at room temperature and at mild pH.

Conditions for the formation of the peptidylthiohydantoins were improved by Stark (1968) and by Dwulet and Gurd (1979), who reported on the use of thiocyanic acid rather than thiocyanate salts, and more recently by studies from our laboratory with the introduction of trimethylsilyl isothiocyanate (TMS-ITC)[1] as a coupling reagent (Hawke et al., 1987). This reagent significantly improved the yields of peptidylthiohydantoin formation and reduced the number of complicating side products. Cleavage of peptidylthiohydantoins by 12 N HCl (Hawke et al., 1987) and by acetohydroxamate (Miller et al., 1989) failed to yield more than a few cycles of degradation.

In addition to the current difficulties associated with cleavage of the derivatized residue, the thiocyanate method also suffers from a lack of reactivity of certain amino acids, in particular aspartate and proline. Whereas some investigators have reported being able to form the thiohydantoin derivatives of these two amino acids, either free in solution or at the C-terminus of a peptide [Yamashita and Ishikawa (1971), Kubo et al. (1971), and more recently Inglis et al. (1989)], others have been unable to obtain any experimental evidence for the formation of either of these two thiohydantoins (Fox et al., 1954; Turner & Schmerzler, 1954; Stark, 1968; Suzuki et al., 1976). Other amino acid side chains that have also been implicated as problematic include arginine, glutamine, glutamate, and lysine (Baptist & Bull, 1953; Fox et al., 1954; Turner & Schmerzler, 1954; Miller et al., 1989).

This study continues work begun in our laboratory concerning the optimization of reaction conditions for C-terminal sequencing with the thiocyanate method by utilizing model peptides containing a variety of C-terminal amino acid side chains to examine conditions for the formation of peptidylthiohydantoins with TMS-ITC and for cleavage of the peptidylthiohydantoin derivatives into a shortened peptide and thiohydantoin amino acid derivative.

Experimental Procedures

Materials. Ultrapure acetic acid was purchased from Alfa Chemicals. Acetic anhydride and pyridine were distilled prior to use. Ammonium thiocyanate and acetohydroxamic acid were from Aldrich. The L-amino acids used for the synthesis of the reference thiohydantoins were all obtained from Sigma Chemical Co. with the exception of N^{α}-acetyl-L-asparagine, which was from Fluka. Water was purified on a Millipore Milli-Q system. Trimethylsilyl isothiocyanate (TMS-ITC) was obtained from Petrarch. All of the peptides used in this study were obtained from either Bachem or Sigma except for Val-Ile-His-Asn-Leu, which was synthesized and purified at the City of Hope Peptide Synthesis Facility. Triethylamine (sequanal grade) was obtained from Pierce.

Preparation of Amino Acid Thiohydantoins. The thiohydantoins derived from alanine, valine, leucine, isoleucine, phenylalanine, methionine, glycine, threonine, lysine, tyrosine, asparagine, glutamine, aspartate, glutamate, arginine, histidine, tryptophan, and *S*-methylcysteine were prepared as described by Suzuki et al. (1976) with only minor modifications. Typically, the amino acid (0.02 mol) was heated at 40 °C in a solution containing acetic anhydride (30 mL) and acetic acid (5 mL) until dissolved. Ammonium thiocyanate (1.8 g, 0.024 mol) was then added and the solution heated for 1 h at 40 °C. The solution was then poured onto 100 g of cracked ice with stirring. The resulting 1-acetyl-2-thiohydantoin was either collected by filtration or evaporated to an oil. Deacetylation was performed by adding acetic acid (1 mL) and 12 N HCl (30 mL) and stirring at room temperature for 2–6 h. The acidified residue was evaporated to dryness under vacuum and recrystalized from water. Aspartate β-methyl ester and glutamate γ-ethyl ester were used for the synthesis of the aspartate and glutamate thiohydantoin derivatives, respectively, in order to avoid unwanted reactions with the side-chain carboxylic group. The synthesis of thiohydantoin arginine was performed at room temperature in order to avoid the formation of triacetylanhydroarginine (Bergmann & Koster, 1926). The reaction with ammonium thiocyanate was allowed to proceed overnight to ensure complete conversion to the thiohydantoin. N^{α}-Acetyl-L-asparagine was used for the synthesis of thio-

[1] Abbreviations: TMS-ITC, trimethylsilyl isothiocyanate; FAB-MS, fast atom bombardment mass spectrometry; MH^+, protonated molecular ion; TFA, trifluoroacetic acid.

Table I: Characterization of the Amino Acid Thiohydantoins by FAB-MS, UV Absorption Spectroscopy, and HPLC

amino acid	FAB $(M + H)^+$	λ_{max}[a] (nm)	retention time[b] (min)
Asn	174	265	3.2
Gly	117	263	3.6
Asp	175	265	4.4
Gln	188	265	5.1
His	197	267	5.5
Ala	131	264	6.5
Glu	189	265	7.8
Arg	216	266	13.8
S-methyl-Cys	177	266	14.5
Thr[c]	143	319	18.2
Lys[d]	230	265	20.0
Val	159	265	20.5
Met	191	266	23.1
Tyr	223	267	23.6
Ile	173	266	29.0[e]
Leu	173	265	32.0
Phe	207	266	34.5
Trp	246	265[f]	38.4

[a] UV absorption spectroscopy was performed on a Beckman Model DU-70 spectrophotometer with methanol as a solvent for the thiohydantoins. [b] HPLC of the amino acid thiohydantoins was performed as described under Experimental Procedures. [c] 5-Ethylidene-2-thiohydantoin was obtained. [d] The side chain ϵ-amino group is acetylated. [e] Appears as a doublet due to racemization about the α-carbon during synthesis leading to the formation of a mixture of L-isoleucine and D-alloisoleucine (Rangarajan et al., 1973). [f] There is a shoulder present at 290 nm.

hydantoin asparagine due to the insolubility of asparagine in acetic anhydride. Attempts to synthesize thiohydantoin proline and serine were unsuccessful.

Characterization of the Amino Acid Thiohydantoins. The recrystallized amino acid thiohydantoins were characterized by HPLC, UV absorption, and FAB-MS (data shown in Table I) as described below. In each case a single peak with a characteristic retention time was observed by HPLC for each thiohydantoin. A single $(M + H)^+$ ion was also observed by FAB mass spectrometry for each amino acid derivative consistent with the expected structure. The UV absorption data was in excellent agreement with that reported by Cromwell and Stark (1969), and extinction coefficients reported by these authors were used in the calculation of stock solutions.

HPLC Separation of Peptides. Reverse-phase HPLC was performed on a Vydac C18 column (2.1 mm × 25 mm) on a Beckman System Gold with a Shimadzu (SPD-6A) detector. Peptide samples were monitored by absorbance at 214 nm and thiohydantoin amino acids at 254 nm. A linear gradient from 2% solvent B to 60% solvent B over 30 min at a flow rate of 0.25 mL/min was employed at room temperature. Solvents A and B, respectively, were 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in 90% acetonitrile/9.9% H_2O.

HPLC Separation of the Amino Acid Thiohydantoins. Reverse-phase HPLC was performed on the system described above with a Beckman ODS Ultrasphere column (2.0 mm × 25 mm). The column was eluted for 6 min with solvent A (0.8% TFA) followed by a linear gradient to 35% solvent B (72% acetonitrile, 8% methanol in water) over 35 min at a flow rate of 0.25 mL/min at 24 °C.

Formation of Peptidylthiohydantoins. The sample peptide (1–60 nmol) in 20 μL (350 μmol) of acetic acid was placed in a 1.5-mL polypropylene tube in which a hole had been bored into the cap, permitting the tube contents to be kept under argon. On addition of acetic anhydride (100 μL, 1058 μmol) the peptide solution was allowed to incubate at 50 °C for 10 min. Trimethylsilyl isothiocyanate (TMS-ITC), 5–50 μL (35–355 μmol) was then added and the solution allowed to incubate at 50 °C for an additional 30 min. The reaction was quenched with water (50 μL) and taken to dryness in a vacuum centrifuge. Dried samples were taken up in water (100 μL) and analyzed by HPLC.

Formation of Peptidylthiohydantoins by Separate Reactions with Acetic Anhydride and TMS-ITC. Sample peptide (30–60 nmol) in 100 μL of acetic anhydride (1058 μmol) was incubated at 50 °C for 10 min a 1.5-mL polypropylene tube and then taken to dryness in a vacuum centrifuge. The peptide was redissolved in anhydrous acetonitrile (50 μL) and 20 μL of TMS-ITC (140 μmol) added. The reaction was incubated for 30 min at 50 °C and then taken to dryness in a vacuum centrifuge. The sample was taken up in water (100 μL) and analyzed by HPLC.

Cleavage of Peptidylthiohydantoins. Cleavage with acetohydroxamic acid was performed at various concentrations and reaction times as indicated in the text. Typically, dried peptidylthiohydantoins (1–20 nmol) were taken up in 50 μL of cleavage solution, incubated at 50 °C for the indicated length of time, and subsequently taken to dryness in a vacuum centrifuge. These samples were redissolved in 40 μL of 25% acetic acid, again taken to dryness, and finally taken up in water for HPLC analysis. For cleavage with triethylamine, the dried peptidylthiohydantoin (0.08–20 nmol) was first dissolved in 100 μL of water, and 2 μL of triethylamine was added (0.14 M). The cleavage solution was then incubated at the indicated temperature and length of time, quenched by addition of acetic acid (5 μL), and dried in a vacuum centrifuge. The cleaved peptides were redissolved in water for HPLC analysis.

Reaction of Peptides with Glycine Ethyl Ester. Peptides were dissolved in 70 μL of acetic anhydride (740 μmol) and incubated at 50 °C for 10 min. At the end of the incubation the acetic anhydride was removed by vacuum centrifugation and the peptide redissolved in 70 μL of acetonitrile. Glycine ethyl ester (6 μmol) dissolved in acetonitrile (30 μL) was added and the reaction allowed to incubate at 50 °C for 30 min. The solvents were evaporated by vacuum centrifugation, and the dried peptide was dissolved in water (100 μL) for analysis by HPLC.

Determination of Rate Constants. Rate constants for cleavage of peptidylthiohydantoins by triethylamine and the cleavage of peptide hydroxamate esters by triethylamine and potassium hydroxide were determined by incubating the peptide for the indicated length of time and quenching with acetic acid. Samples were then dried in a vacuum centrifuge and redissolved in water for quantitation of the remaining uncleaved peptide by HPLC. The rate of hydrolysis was determined from a semilogarithmic plot of C/C_0 as a function of time, where C_0 represents the initial concentration of unhydrolyzed peptide and C represents the concentration of unhydrolyzed peptide at a given time. Pseudo-first-order rate constants were calculated from a linear least-squares fit to the data.

Mass Spectrometry. Positive ion fast atom bombardment mass spectrometry (FAB-MS) was performed as described by Hefta et al. (1988). Peptide samples from HPLC were collected in 1.5-mL polypropylene tubes, dried in a vacuum centrifuge, and redissolved in 2–3 μL of 5% aqueous acetic acid. An aliquot (1–2 μL) of the peptide was added directly to 2–3 μL of glycerol on the sample stage. The amino acid thiohydantoins were dissolved in methanol (typically 2–3 mg in 200 μL of methanol), and 2 μL of this solution was added directly to the glycerol on the sample stage. Data were collected with a JEOL HX-100HF mass spectrometer operating

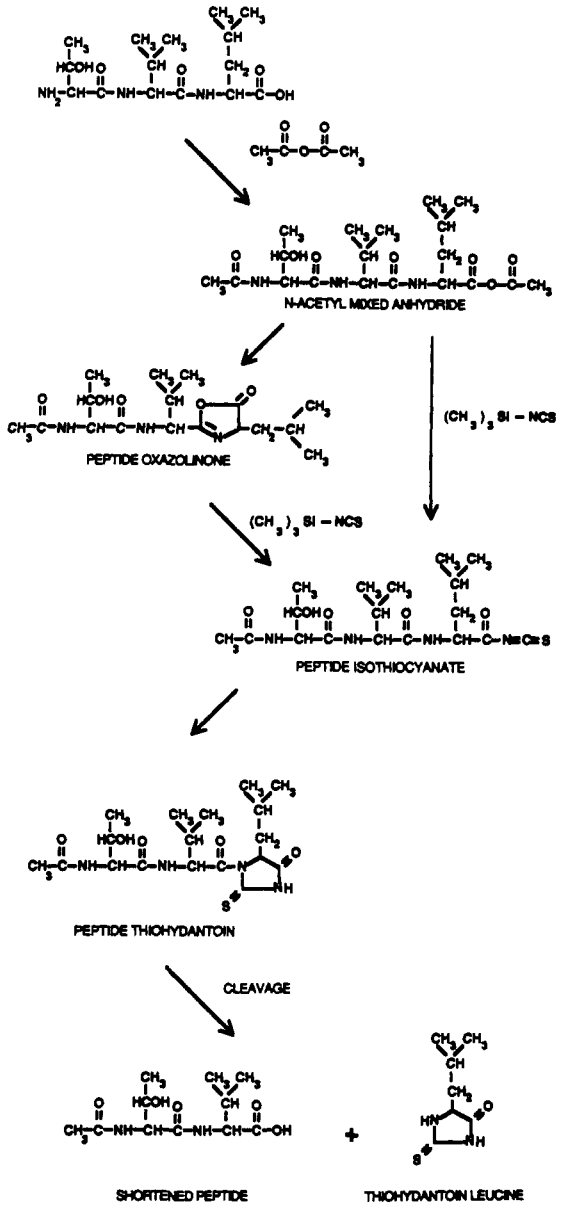

FIGURE 1: Reaction scheme for the degradation of schizophrenia-related peptide using thiocyanate chemistry.

at a 5-kV accelerating potential. The daughter ions resulting from decomposition from a selected ion (in this case, MH^+ = 415) were examined by *B/E*-linked scans (Wetson et al., 1976), where the electric sector voltage and magnetic sector field strength were linked such that the ratio was constant. For normal spectra, the electric sector was set at a constant value (5 kV) and the magnetic sector was scanned over a given mass range.

Amino Acid Analysis. Peptide samples purified by HPLC were dried in a hydrolysis tube in a vacuum centrifuge and redissolved in 300 μL of 6 N HCl containing 0.2% β-mercaptoethanol. Hydrolysis in this solution was performed under vacuum for 22 h at 110 °C. Compositional analysis was performed on a Beckman system 6300 amino acid analyzer.

Results

Reaction of Schizophrenia Peptide with TMS-ITC. The reaction of schizophrenia-related peptide (NH_2-Thr-Val-Leu-COOH) with TMS-ITC in acetic anhydride is shown in Figure 1. Analysis of this reaction mixture by HPLC and FAB-MS reveals the presence of four peptidylthiohydantoins (Figure 2, peaks b–e) and some underivatized *N*-acetyl peptide

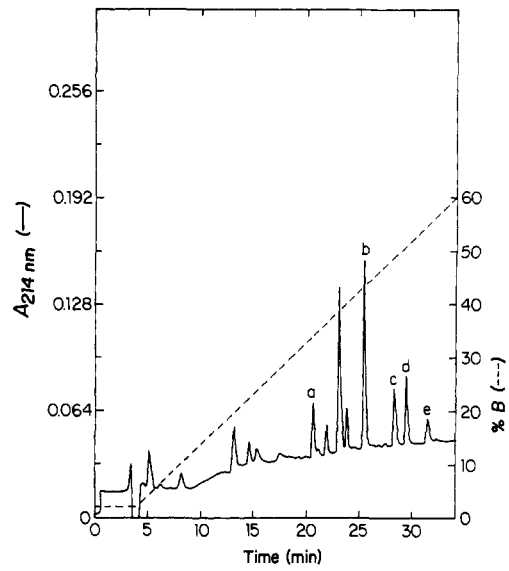

FIGURE 2: HPLC analysis of model peptidylthiohydantoins. Schizophrenia-related peptide (Thr-Val-Leu, 50 nmol) in 20 μL of acetic acid (350 μmol) and 100 μL of acetic anhydride (1058 μmol) was reacted with 20 μL of TMS-ITC (142 μmol) for 30 min at 50 °C. The reaction was quenched with H_2O, dried down, and redissolved in 100 μL of H_2O. An aliquot (14 μL, 3.6 nmol) was analyzed by HPLC as described under Experimental Procedures. Absorbance was monitored at 214 nm at 0.32 AUFS. Peak a is *N*-acetyl-Thr-Val-Leu (14% of total peptide products), which do not react under these conditions to form peptidylthiohydantoins. Peaks b–e are all peptidylthiohydantoins. The differences between these peptidylthiohydantoins are discussed in the text. The remaining peaks were present when a control reaction without any peptide was analyzed (data not shown), indicating that these peaks are due to the reagents used for the reaction.

(peak a). HPLC analysis of a control reaction containing only TMS-ITC and acetic anhydride revealed the remaining peaks to be nonpeptide products. Other experiments have shown that formation of the peptidylthiohydantoin derivatives can be driven to completion by either addition of more TMS-ITC (30 μL rather than 20 μL), addition of a catalyst such as pyridine (0.225 M; Hawke et al., 1987), or a longer reaction time (45 min). Reaction of small quantities of schizophrenia peptide (1 nmol) with TMS-ITC (142 μmol) gave the same distribution of peptide products shown in Figure 2, indicating that this chemistry works equally as well on sample quantities ranging from 1 to 60 nmol. Amino acid composition analysis of each of the four peptidylthiohydantoin peaks (data not shown) gave identical results with the expected 1:1:1 ratio of Thr, Val, and Leu found for each one. Peaks b and c were shown by FAB-MS (data not shown) to have an MH^+ ion of 415 corresponding to the expected *N*-acetylpeptidylthiohydantoin (Figure 1). Peaks d and e had an MH^+ of 457, indicating that these two peaks were also *N*-acetylpeptidylthiohydantoins with an additional acetyl group on the threonine hydroxyl. Examination of the daughter ions resulting from both MH^+ 415 peaks (b and c) by linked-scan FAB-MS showed fragment ions consistent with the expected structure (Figure 3). The spectra differ only in the relative amounts of the fragment ions. Incubation of these two peptidylthiohydantoins (peaks b and c), individually, under the exact conditions used for their synthesis and subsequent analysis by HPLC showed that these peptides are not interconvertible under the conditions used for their formation, indicating that the differences between these two peptidylthiohydantoins must arise during the reaction with TMS-ITC. Variation of the concentration of TMS-ITC used in the reaction from 1.0 to 4.4 M (20–200 μL) only slightly increased the ratio of peaks

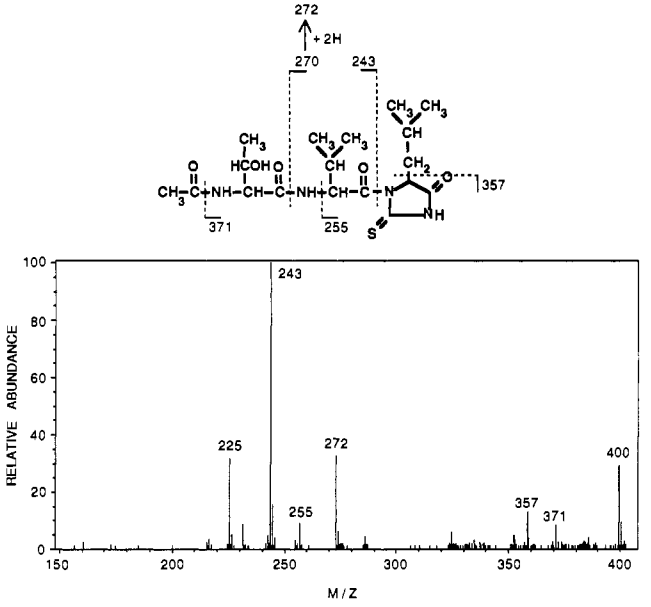

FIGURE 3: Analysis of model peptidylthiohydantoins by FAB-MS. Mass spectrum showing the ions resulting from a *B/E*-linked scan from $MH^+ = 415$ for peptidylthiohydantoin peak b (1.5 nmol of sample was used). An identical scan using the other peptidylthiohydantoin of $MH^+ = 415$ (peak c) resulted in identical fragment ions (data not shown). The only difference was that the intensity of the 272 peak was now equal to the intensity of the 243 peak.

b to c and peaks d to e. Inclusion of pyridine (0.225 M) in the reaction mixture as a catalyst (Hawke et al., 1987) produced only thiohydantoin peaks d and e. The acetylation of amino acid side chains under these conditions is not unexpected and was originally shown by Dakin and West (1928) to be catalyzed by pyridine. In the presence of pyridine the second peak (peak e) of the pair is now the major peak, rather than the first peak (peak d) in the absence of pyridine. Catalysis with triethylamine (0.103 M), unlike pyridine, gave only the unacetylated threonine peptides (peaks b and c) and did not change the ratio of these two peaks. Removal of acetic acid from the coupling reaction had no effect on the yield, distribution, or rate of formation of the peptidylthiohydantoins. Separation of the activation from the coupling step (details under Experimental Procedures) by first reaction of Thr-Val-Leu with acetic anhydride followed by evaporation and then addition of TMS-ITC in acetonitrile effectively formed thiohydantoin peptides b and c (from Figure 2) in 82% and 11% yields, respectively, with the remaining 7% of recovered peptides being underivatized *N*-acetyl-Thr-Val-Leu.

Cleavage of Peptidylthiohydantoins with Acetohydroxamate. Cleavage of peptidylthiohydantoins by aqueous acetohydroxamate under basic conditions was first proposed by Stark (1968) and further studied by Meuth et al. (1982) and Miller et al. (1989). The advantages of this reagent as first proposed by Stark (1968) are the mild conditions required for the selective hydrolysis of the C-terminal thiohydantoin amino acid as compared to hydrolysis by 12 N hydrochloric acid (Cromwell & Stark, 1969) or 1 N sodium hydroxide (Schlack & Kumpf, 1926), both of which in addition to cleavage at the C-terminal thiohydantoin amide bond can also cause hydrolysis of internal protein (peptide) amide bonds.

Cleavage of peptidylthiohydantoins (peak b, Figure 2) by 0.1 M acetohydroxamate, pH 8.2, in 50% pyridine, conditions similar to those used by Stark (1968), for 60 min at 50 °C gave the results shown in Figure 4A. Two peptide products were obtained, peaks I and II. Peak I was found to have an MH^+ ion of 261, consistent with the expected *N*-acetyl-Thr-Val-COOH peptide, and peak II had an MH^+ ion of 318, indicating that this peptide was derivatized at the C-terminus by acetohydroxamate, forming a hydroxamate ester as shown in Figure 5. Peak III was identified as thiohydantoin leucine by FAB-MS ($MH^+ = 173$) and by its identical retention time with a thiohydantoin leucine standard. The remainder of the peaks are due to breakdown products of thiohydantoin leucine (data not shown). The extensive breakdown of thiohydantoin leucine may have been due to the higher pH caused by including pyridine, thereby facilitating reaction of acetohydroxamate at one or more of the electrophilic centers on the

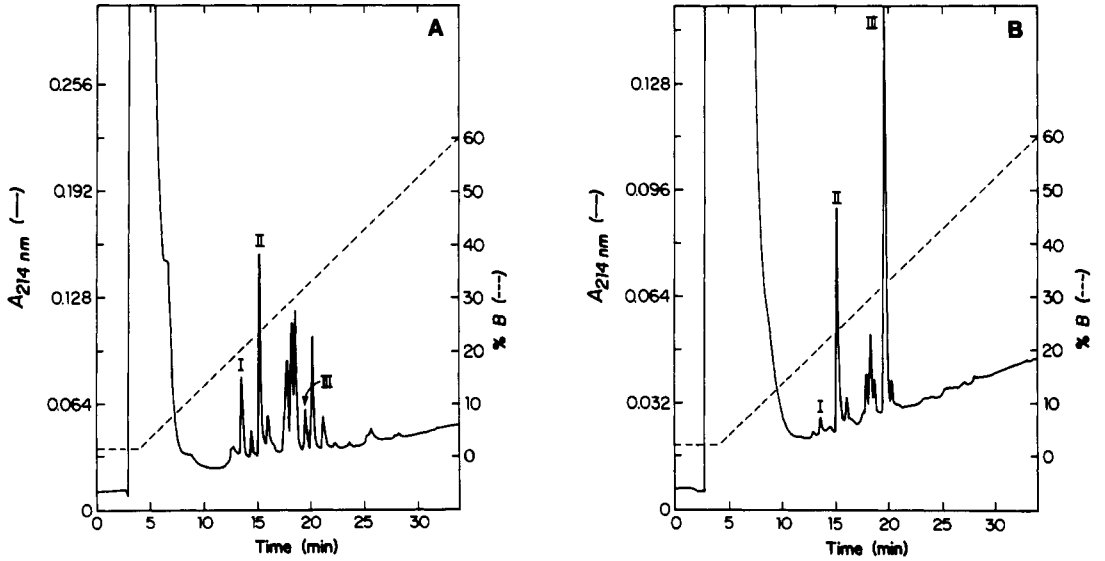

FIGURE 4: HPLC analysis of model peptidylthiohydantoin hydrolyzed with acetohydroxamate. (A) Peptidylthiohydantoin (9 nmol, peak b from Figure 2) was reacted with 0.1 M acetohydroxamate, pH 8.2, in 50% pyridine for 60 min at 50 °C. The reaction was dried down, acidified with acetic acid, and redissolved in 100 μL of H_2O. An aliquot (4 nmol) was analyzed by HPLC as described under Experimental Procedures. Absorbance was monitored at 214 nm at 0.32 AUFS. Peak I (32% of the total peptide products) is the shortened peptide *N*-acetyl-Thr-Val, and peak II is also the shortened peptide, *N*-acetyl-Thr-Val, but is derivatized to a hydroxamate ester at the C-terminus by acetohydroxamate. Peak III is thiohydantoin leucine. The remainder of the unlabeled peaks are breakdown products derived from thiohydantoin leucine. (B) Peptidylthiohydantoin (1.5 nmol) was reacted with 0.1 M acetohydroxamate, pH 8.2, for 30 min at 50 °C. The reaction mixture was dried, acidified with acetic acid, taken up with 100 μL of H_2O, and analyzed by HPLC as described above. Peaks I (7% of total peptide products), II, and III correspond to the same peaks as above.

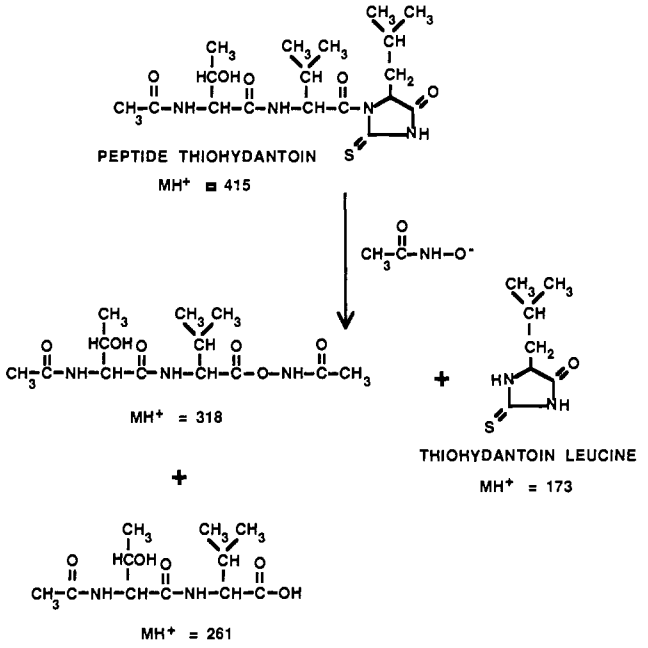

FIGURE 5: Reaction scheme for the hydrolysis of peptidylthiohydantoin with acetohydroxamate.

thiohydantoin ring. The acetohydroxamate solution was made to pH 8.2 prior to addition of pyridine, which raised the pH to 9.8. It is likely that acetohydroxamate, which has a pK of 9.4 (Stark, 1968), may facilitate breakdown of thiohydantoin leucine at this higher pH. Reduction of the reaction time for cleavage from 60 to 30 min significantly reduced the breakdown of thiohydantoin leucine (data not shown). A second cycle of degradation was attempted on both peptide peaks I and II (Figure 4A). Peptide I on reaction with acetic anhydride and TMS-ITC gave the expected thiohydantoin peptide, *N*-acetyl-Thr-thiohydantoin-Val ($MH^+ = 302$) in quantitative yield, whereas reaction of peak II under the same conditions, in the presence of a pyridine catalyst, gave only peptide with two additional acetyl groups (presumably one of these acetyl groups is on the threonine hydroxyl and the other on the acetohydroxamate nitrogen) and no thiohydantoin peptide. When the reaction time for acetohydroxamate cleavage was reduced from 60 to 30 min to minimize degradation of thiohydantoin leucine, the amount of underivatized peptide (peak I) was reduced from 32% to 25% of the total peptide products. Removal of pyridine from the cleavage solution, while lowering the pH and thus significantly minimizing any breakdown of thiohydantoin lecuine, reduced the amount of underivatized peptide (peak I, *N*-acetyl-Thr-Val-COOH) to only 7% of the total peptide products (Figure 4B). Increase of the acetohydroxamate concentration up to 0.8 M had no effect on the distribution of peptide products. These results suggested that the more basic reaction conditions when pyridine was present may have allowed some hydrolysis of the hydroxamate ester to give the desired C-terminal carboxylic acid. This is consistent with observations by Stieglitz and Leech (1914) and by Scott and Mote (1927), who isolated esters of hydroxamic acids and found them to undergo rearrangement to the free carboxylic acid under basic conditions. Incubation of the peptidyl hydroxamate ester formed in this study (peak II, Figure 4A) in 0.2 N hydrochloric acid for 30 min at 50 °C resulted in no hydrolysis to the free carboxylic acid. Examination of the kinetics of hydrolysis of peak II (Figure 4A) by 2% triethylamine (0.14 M, pH 11.5) at 50 and at 22 °C gave pseudo-first-order rate constants of 0.015 min^{-1} and 0.0018 min^{-1}, respectively, with half-times of 46 and 385 min (Figure 6). Hydrolysis of this peptidyl hydroxamate ester to the free carboxylic acid by 0.14 M potassium hydroxide at 50 °C gave a pseudo-first-order rate constant of 0.083 min^{-1} with a half-time of 8 min. As hydrolysis of the peptidyl hydroxamate ester is faster with an equivalent concentration of potassium hydroxide than triethylamine, it is possible that the potassium cation is participating in the rearrangement, as suggested by Scott and Mote (1927). Cleavage of peptidylthiohydantoin peak c (Figure 2) by acetohydroxamate under identical conditions as peak b gave the same ratio of products as found with peak b, indicating that cleavage of the thiohydantoin amino acid removes the differences between these

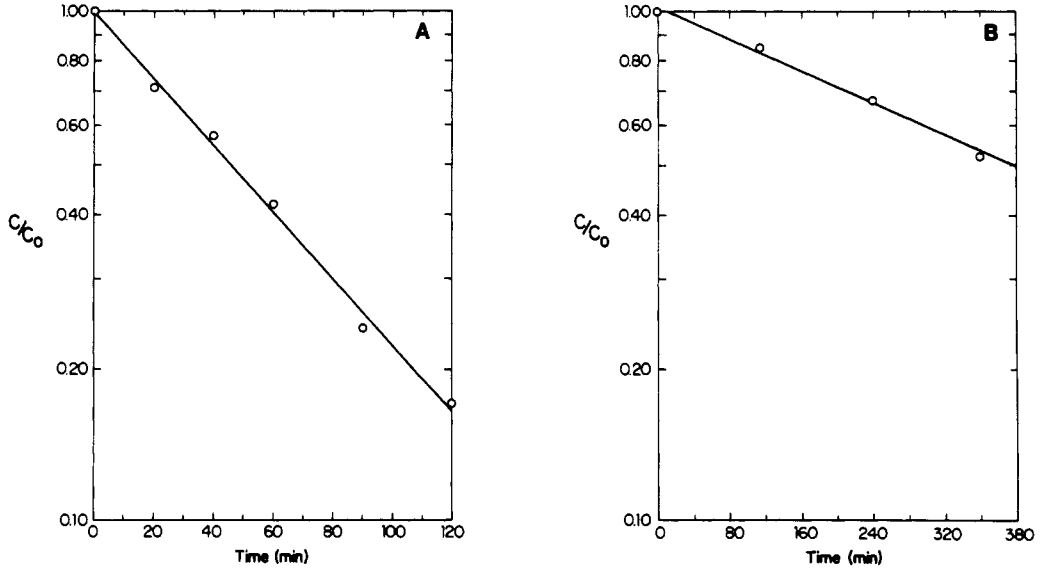

FIGURE 6: Kinetic analysis for the hydrolysis of a model peptidyl hydroxamate ester by aqueous 2% triethylamine. (A) Peptidyl hydroxamate ester (peak II, Figure 4) (12 nmol) was dissolved in 160 μL of H_2O and divided into eight 20-μL aliquots (1.5 nmol of peptide each). Each aliquot was taken up to a total volume of 100 μL with H_2O, and 2 μL of triethylamine was added. The peptides were incubated at 50 °C for the indicated times. The reaction was quenched by the addition of acetic acid (5 μL) and dried in a vacuum centrifuge. The peptide was taken up in 100 μL of H_2O and analyzed by HPLC. A pseudo-first-order rate constant, based on the percentage of unhydrolyzed peptidyl hydroxamate ester remaining, of 0.015 min^{-1} with a half-time of 46 min was calculated as described under Experimental Procedures. (B) Peptidyl hydroxamate ester was hydrolyzed with aqueous 2% triethylamine exactly as described above with the single exception that the hydrolysis was carried out at 22 °C. A pseudo-first-order rate constant of 0.0018 min^{-1} with a half-time of 385 min was calculated.

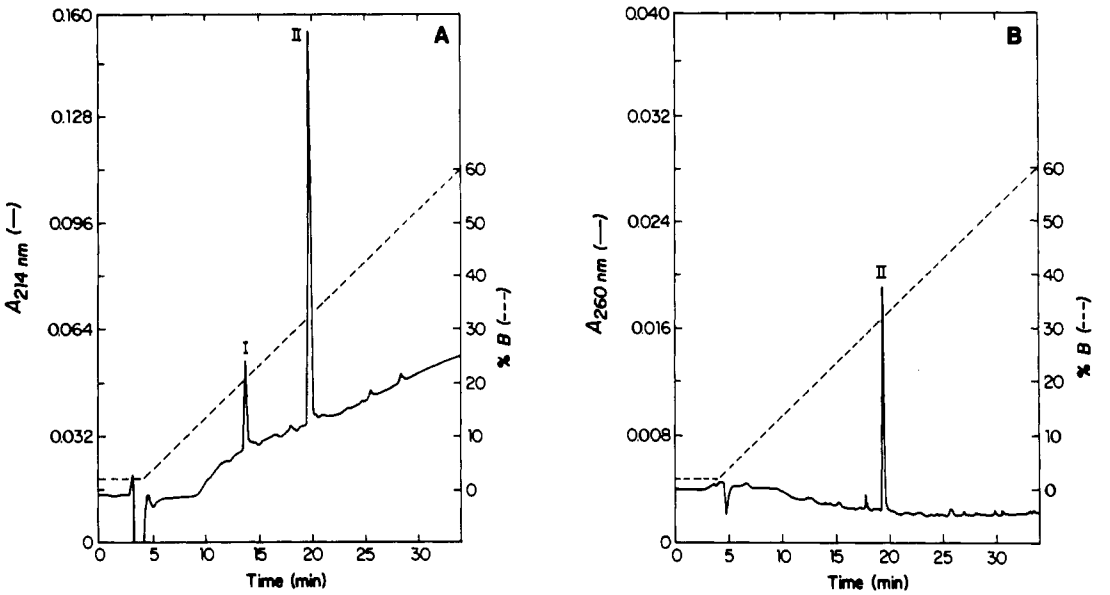

FIGURE 7: HPLC analysis of model peptidylthiohydantoin hydrolyzed with aqueous 2% triethylamine. (A) Peptidylthiohydantoin (peak b, Figure 2) (1.1 nmol) was hydrolyzed with aqueous 2% triethylamine (0.14 M, pH 11.5) at 50 °C for 5 min. The reaction was quenched by addition of acetic acid (5 μL) and dried in a vacuum centrifuge. The hydrolyzed peptide was dissolved in 100 μL of H_2O and analyzed by HPLC as described under Experimental Procedures. Absorbance was monitored at 214 nm at 0.16 AUFS. Peak I is the shortened peptide, *N*-acetyl-Thr-Val, and peak II is thiohydantoin leucine. (B) Peptidylthiohydantoin (80 pmol) was hydrolyzed with aqueous 2% triethylamine and analyzed by HPLC exactly as described above. In this case, the absorbance was monitored at 260 nm at 0.04 AUFS, allowing only thiohydantoin leucine to be observed.

two peptidylthiohydantoins. Cleavage of peptidylthiohydantoin peaks d and e (Figure 2) also gave identical products. Reaction of peaks d and e with acetohydroxamate, while removing the thiohydantoin amino acid, did not remove the *O*-acetyl group from threonine.

Cleavage of Peptidylthiohydantoins with Triethylamine. Although cleavage of peptidylthiohydantoins by aqueous triethylamine has been reported by several authors (Kubo et al., 1971; Dwulet & Gurd, 1979; Meuth et al., 1982), use of this reagent appears never to have been pursued. Cleavage of peptidylthiohydantoin (peak b, Figure 2) by 2% aqueous triethylamine (pH 11.5) is shown in Figure 7. Peak I is the expected *N*-acetyl-Thr-Val-COOH peptide ($MH^+ = 261$), and peak II is thiohydantoin leucine ($MH^+ = 173$). Under the conditions used here, 1 nmol of peptide incubated at 50 °C for 5 min, no degradation of thiohydantoin leucine is evident. Pseudo-first-order rate constants for the cleavage of peptidylthiohydantoin by 2% aqueous triethylamine at 37 and 22 °C were calculated to be 0.49 min^{-1} and 0.15 min^{-1}, respectively, with half-times of 1 and 5 min. Cleavage of all the thiohydantoin peptides in Figure 2 (peaks b–e) gave identical products, indicating that the *O*-acetyl group on the threonine side chain (peaks d and e) was simultaneously removed with cleavage of the C-terminal thiohydantoin amino acid and that, during sequential degradation using triethylamine as a cleavage reagent, all of the thiohydantoin peptides observed were equivalent. It is also interesting to note that during the kinetic study of hydrolysis of the C-terminal thiohydantoin amino acid by 2% aqueous triethylamine the uncleaved peptidylthiohydantoin, present at the earlier time points, was recovered as two peaks regardless of which peptidylthiohydantoin peak (b or c) was originally chosen. This indicates that under these basic conditions of cleavage peptidylthiohydantoin (peaks b and c) can be easily interconverted. The cleavage of 80 pmol of peptidylthiohydantoin by 2% aqueous triethylamine is shown in Figure 7B, demonstrating the utility of triethylamine cleavage even at the subnanomole level.

Reaction of TMS-ITC with Peptides Containing Different C-Terminal Amino Acids. Peptides containing a variety of C-terminal amino acids (Table II) were reacted with TMS-ITC in order to examine any problems that might arise during a sequential degradation utilizing the thiocyanate chemistry. In all, 15 different amino acids were examined. In each case the derivatized peptides were separated by HPLC and examined by FAB-MS. As with Thr-Val-Leu, two peptidylthiohydantoins were found for each peptide, except for when the C-terminal amino acid was glycine (Val-Gly-Val-Ala-Pro-Gly). Derivatization of peptides containing the C-terminal residues Leu, Val, Phe, Gln, Asn, Gly, Lys, Arg, Ser, Trp, and His was easily accomplished. Problems were found with Glu, Asp, Thr, and Pro.

Reaction of C-terminal glutamate (Phe-Leu-Glu-Glu) with TMS-ITC (140 μmol) as described under Experimental Pro-

Table II: Peptides Used for Degradation with TMS-ITC and Aqueous Triethylamine[a]

peptide	cycles performed	residues cleaved[b]
Thr-Val-Leu	2	Leu, Val
Try-Gly-Gly-Phe-Leu	2	Leu, Phe
Trp-His-Trp-Leu-Gln-Leu	2	Leu, Gln
Phe-Leu-Glu-Glu-Leu	2	Leu, Glu
Val-Ile-His-Asn-Leu	2	Leu, Asn
p-Glu-Lys-Trp-Ala-Pro	no rctn[c]	none
Val-Gly-Val-Ala-Pro	no rctn	none
Ala-Phe-Pro	no rctn	none
Val-Gly-Val-Ala-Pro-Gly	1	Gly
Val-Ile-His-Asn	2	Asn, His
Tyr-Thr-Gly-Phe-Leu-Thr	1	Thr
Val-Gly-Ser-Glu	1	Glu
Pro-Phe-Asp	no rctn	none
Tyr-Gly-Gly-Phe-Leu-Lys	1	Lys
Tyr-Gly-Gly-Phe-Leu-Arg	1	Arg
Thr-Tyr-Ser	1	Ser
Tyr-Lys-Trp	1	Trp

[a] Each peptide (40–60 nmol) was reacted with TMS-ITC (140 μmol) in the presence of acetic anhydride (1058 μmol) as described under Experimental Procedures. Hydrolysis of the peptidylthiohydantoins into a shortened peptide and amino acid thiohydantoin was accomplished by incubation with 2% aqueous triethylamine for 10 min at 50 °C as described under Experimental Procedures. [b] Unless otherwise stated, yields were in the range of 90–100%. [c] No reaction.

cedures resulted in derivatization of 35% of the starting peptide to the peptidylthiohydantoin. The remainder of the peptide was recovered as *N*-acetyl peptide. Inclusion of either triethylamine (0.103 M) or pyridine (0.225 M) catalysts during the reaction with TMS-ITC resulted in 0% and 9% formation of peptidylthiohydantoin, respectively, on the basis of the amount of peptides recovered. An increase in the amount of TMS-ITC (355 μmol) used in the reaction raised the percent derivatization to peptidylthiohydantoin to 54%. Reaction of the peptide Val-Gly-Ser-Glu with TMS-ITC by the alternate procedure described under Experimental Procedures involving a separate activation with acetic anhydride and followed by a separate reaction with TMS-ITC resulted in an 89% derivatization to the peptidylthiohydantoin. This is an equivalent yield of peptidylthiohydantoin to that obtained with the Thr-Val-Leu peptide under identical conditions.

Reaction of Pro-Phe-Asp with TMS-ITC under the conditions used above with the C-terminal glutamate peptides failed to yield any peptidylthiohydantoins. As with glutamate, reaction of aspartate with acetic anhydride is known to form a five-membered cyclic anhydride rather than an oxazolinone, and unlike the oxazolinone the resulting cyclic anhydrides are well documented not to be reactive toward ammonium thiocyanate (Nicolet, 1930; Swan, 1952; Barker, 1953; Stark, 1968). Reaction of *N*-acetylaspartic acid anhydride with aniline was found to give two isomeric anilides, one capable of reaction with ammonium thiocyanate to form a thiohydantoin and the other incapable of any reaction (Swan, 1952). Such an approach was tried here with *N*,*N*-dimethylethylenediamine (Figure 8) and glycine ethyl ester. The reaction of Pro-Phe-Asp with *N*,*N*-dimethylethylenediamine (Figure 8) was performed as described in the figure legend. The reaction products were analyzed by HPLC and FAB-MS as described under Experimental Procedures. The reaction was quantitative, with only two separable peaks obtained. Both peaks had a MH^+ ion of 490 as expected for the two possible isomers on reaction with *N*,*N*-dimethylethylenediamine. One isomer accounted for 62% of the total peptide products and the other 38%. When these two peptides were individually reacted with TMS-ITC as described under Experimental Procedures, both gave quantitatively the same succinimide product (MH^+ = 472) shown in Figure 8. No peptidylthiohydantoins could be detected. Reaction of Pro-Phe-Asp with acetic anhydride and glycine ethyl ester as described under Experimental Procedures gave quantitatively the expected product (MH^+ = 505). Although two isomers were probably formed as with the reaction with *N*,*N*-dimethylethylenediamine, they were not resolved by HPLC as only a single peak was obtained. Treatment of this product with acetic anhydride and TMS-ITC resulted in a 58% conversion to the expected peptidylthiohydantoin (MH^+ = 546). The yield of peptidylthiohydantoin could not be pushed higher by longer reaction times, suggesting that the reaction of glycine ethyl ester with peptidylaspartic acid anhydride probably formed both isomers as expected and that the reaction of glycine ethyl ester (and possibly *N*,*N*-dimethylethylenediamine) with *N*-acetylaspartic acid anhydride is slightly favored at the β-carbonyl group (60% vs 40%).

Reaction of Tyr-Thr-Gly-Phe-Leu-Thr with acetic anhydride and TMS-ITC produced two types of peptides, the expected *N*-acetylpeptidylthiohydantoin (MH^+ = 784; 56% yield) and a peptide with an MH^+ = 725 (24% yield), consistent with dehydration of a threonine residue and no thiohydantoin formation. The remaining 20% was underivatized *N*-acetyl peptide (MH^+ = 743). We conclude that the C-terminal threonine residue, and not the internal threonine, was

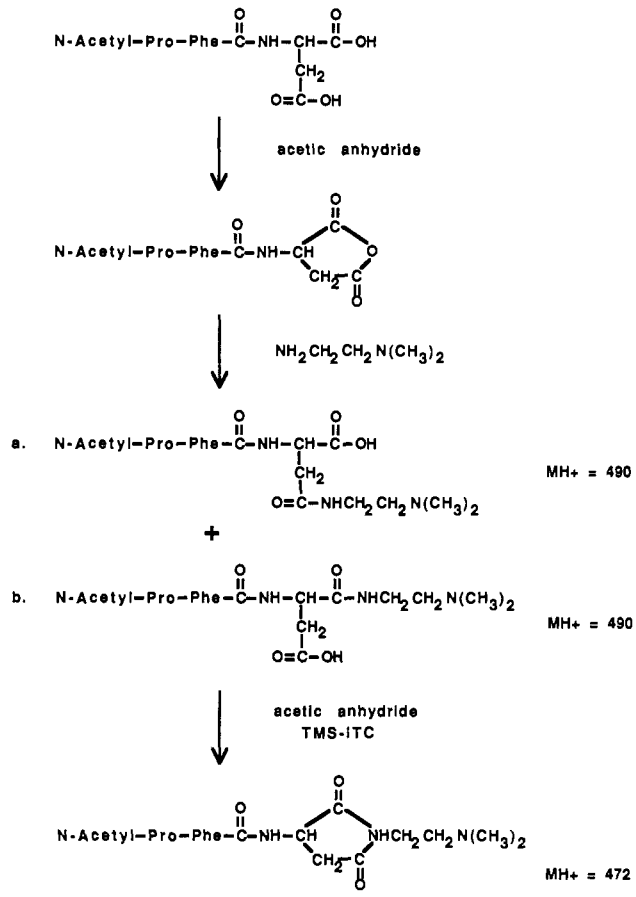

FIGURE 8: Reaction of Pro-Phe-Asp with acetic anhydride and *N*,*N*-dimethylethylenediamine. The tripeptide Pro-Phe-Asp (60 nmol) was reacted with acetic anhydride (740 μL) for 10 min at 50 °C. At the end of the incubation the acetic anhydride was removed by vacuum centrifugation. *N*,*N*-Dimethylethylenediamine (46 μmol) in 30 μL of acetonitrile was added and the reaction allowed to incubate at 50 °C for 30 min. The reaction was again dried by vacuum centrifugation and analyzed by HPLC as described under Experimental Procedures. Peptides a and b were reacted with TMS-ITC (140 μmol) in the presence of acetic anhydride (1058 μmol) for 30 min at 50 °C as described under Experimental Procedures.

the dehydrated derivative since the threonine residue in the peptides Thr-Val-Leu and Thr-Tyr-Ser was found not to be dehydrated under these reaction conditions. The peptide Tyr-Thr-Gly-Phe-Leu-dehydro-Thr was found to be incapable of forming peptidylthiohydantoins when reacted with acetic anhydride and TMS-ITC. Inclusion of triethylamine (0.103 M) as a catalyst in the reaction of TMS-ITC and Tyr-Thr-Gly-Phe-Leu-Thr did not reduce the amount of dehydro-Thr peptide formed. Reaction of Tyr-Thr-Gly-Phe-Leu-Thr with TMS-ITC in acetonitrile after a separate activation with acetic anhydride as described under Experimental Procedures resulted in a 77% formation of peptidylthiohydantoin, with the remaining 23% being underivatized *N*-acetyl peptide. No evidence of the dehydro-Thr peptide could be found when peptide was reacted under these conditions.

The reactions of peptides containing a C-terminal proline (p-Glu-Lys-Trp-Ala-Pro, Val-Gly-Val-Ala-Pro, and Ala-Phe-Pro) with TMS-ITC in acetic anhydride all failed to yield peptidylthiohydantoins. Inclusion of the catalysts triethylamine and pyridine had no effect. In all reactions analyzed only *N*-acetyl peptide was recovered. In order to determine whether the problem with proline lies with the activation step or the coupling step, we compared the reaction of Thr-Val-Leu and Ala-Phe-Pro with glycine ethyl ester (described under Experimental Procedures). Analysis of the reaction of Thr-Val-Leu with glycine ethyl ester by HPLC revealed two peaks

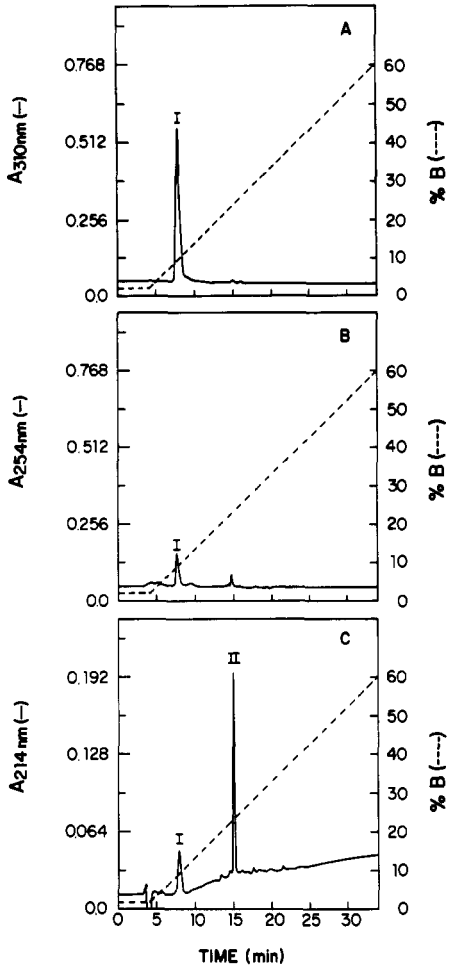

FIGURE 9: HPLC analysis of *N*-acetyl-Thr-Tyr-thiohydantoin-Ser hydrolyzed with 2% aqueous triethylamine. *N*-Acetyl-Thr-Tyr-thiohydantoin-Ser (2.5 nmol) was hydrolyzed for 10 min at 50 °C with 2% aqueous triethylamine (0.14 M, pH 11.5). The reaction was quenched with acetic acid (5 μL) and dried in a vacuum centrifuge. The hydrolyzed peptide was dissolved in 100 μL of H_2O and divided into three aliquots. Each aliquot was analyzed by HPLC as described under Experimental Procedures. (A) HPLC analysis was performed at 310 nm at 1.28 AUFS. (B) Analysis at 254 nm at 1.28 AUFS. (C) Analysis at 214 nm at 0.32 AUFS. Peak I is 5-methylene-2-thiohydantoin, and peak II is the shortened peptide *N*-acetyl-Thr-Tyr-COOH (MH^+ = 325).

each with an MH^+ of 459 consistent with the expected product, Thr-Val-Leu-Gly-OEt, while reaction of Ala-Phe-Pro under identical conditions gave only a single peak with an MH^+ of 461 consistent with the formation of Ala-Phe-Pro-Gly-OEt. Therefore, it is likely that while reaction with Thr-Val-Leu results in the formation of a C-terminal oxazolinone, thereby leading to racemization of the C-terminal amino acid, the reaction of acetic anhydride with C-terminal proline probably forms the linear mixed anhydride (Figure 1).

General Hydrolysis of Peptidylthiohydantoins with Aqueous Triethylamine. Cleavage with 2% aqueous triethylamine was performed for 10 min at 50 °C as described under Experimental Procedures. Each reaction (Table II) was analyzed by HPLC, and the purified peptides were examined by FAB-MS. Peptides containing C-terminal thiohydantoin-Leu, -Val, -Phe, -Gln, -Glu, -Gly, -His, -Thr, -Ser, -Lys, -Arg, and -Trp were all successfully hydrolyzed to an amino acid thiohydantoin and a new shortened peptide. Peptides containing C-terminal Thr and Ser residues were found to be dehydrated during cleavage by aqueous triethylamine. The amino acid thiohydantoin derivative from Tyr-Thr-Gly-Phe-Leu-thiohydantoin-Thr had an absorbance maximum consistent with 5-ethylidene-2-thiohydantoin as well

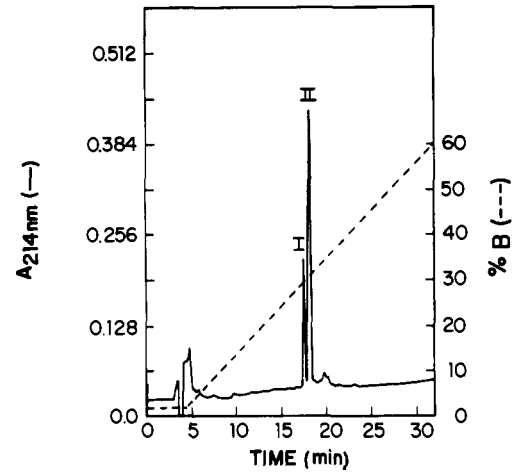

FIGURE 10: HPLC analysis of *N*-acetyl-Val-Ile-His-thiohydantoin-Asn with 2% aqueous triethylamine. *N*-Acetyl-Val-Ile-His-thiohydantoin-Asn (3 nmol) was hydrolyzed for 10 min at 50 °C with 2% aqueous triethylamine. The reaction was quenched with acetic acid (5 μL) and dried in a vacuum centrifuge. The hydrolyzed peptide was dissolved in 100 μL of water and analyzed by HPLC as described under Experimental Procedures. Absorbance was monitored at 214 nm at 0.64 AUFS. Peak I (MH^+ = 409, 31%) is *N*-acetyl-Val-Ile-His-NH_2, and peak II (MH^+ = 410, 69%) is *N*-acetyl-Val-Ile-His-COOH.

as an identical retention time on HPLC as the synthetic standard. The amino acid thiohydantoin obtained from Thr-Tyr-thiohydantoin-Ser (Figure 9) also had a maximum absorbance consistent with an exocyclic double bond (5-methylene-2-thiohydantoin), although no synthetic standard was available for confirmation. The C-terminal aspartylglycine ethyl ester thiohydantoin formed from the reaction of *N*-acetyl-Pro-Phe-Asp-Gly-OEt with TMS-ITC was also easily hydrolyzed, forming the new peptide *N*-acetyl-Pro-Phe-COOH (MH^+ = 305) in quantitative yield.

The only amino acid side chain found to cause problems with cleavage by aqueous triethylamine is asparagine. Hydrolysis of *N*-acetyl-Val-Ile-His-thiohydantoin-Asn (MH^+ = 565) with 2% aqueous triethylamine for 10 min at 50 °C generated two peaks (Figure 10). Peak I (31%) had an MH^+ = 409 and peak II (69%) had an MH^+ = 410, consistent with the expected shortened peptide, *N*-acetyl-Val-Ile-His-COOH. Peak II when reacted with acetic anhydride and TMS-ITC quantitatively formed *N*-acetyl-Val-Ile-thiohydantoin-His (MH^+ = 451), whereas peak I when reacted with TMS-ITC under identical conditions remained unchanged. Since peak I does not react with TMS-ITC and has a molecular ion (MH^+ = 409) consistent with an amidated C-terminus, we concluded peak I to be *N*-acetyl-Val-Ile-His-NH_2. Reaction of *N*-acetyl-Tyr-Gly-Gly-Phe-Leu-NH_2 with TMS-ITC also failed to react with TMS-ITC, confirming the lack of reaction of C-terminal amides with TMS-ITC. Hydrolysis of *N*-acetyl-Val-Ile-His-Asn-thiohydantoin-Leu (MH^+ = 678) with 2% aqueous triethylamine at 50 °C for 10 min quantitatively formed thiohydantoin leucine and two shortened peptides (Figure 11). The first (36%) had an MH^+ = 524, consistent with *N*-acetyl-Val-Ile-His-Asn-COOH, and the second (64%) had an MH^+ = 506, consistent with the succinimide structure shown in Figure 11. This second peak (MH^+ = 506) remained unchanged on reaction with TMS-ITC and acetic anhydride. Hydrolysis of *N*-acetyl-Val-Ile-His-Asn-thiohydantoin-Leu with 2% aqueous triethylamine at 25 °C (instead of 50 °C) resulted in an almost quantitative formation of peak II (94%).

Discussion

Previous studies in this laboratory (Hawke et al., 1987) have focused on a new reagent for thiocyanate chemistry. This

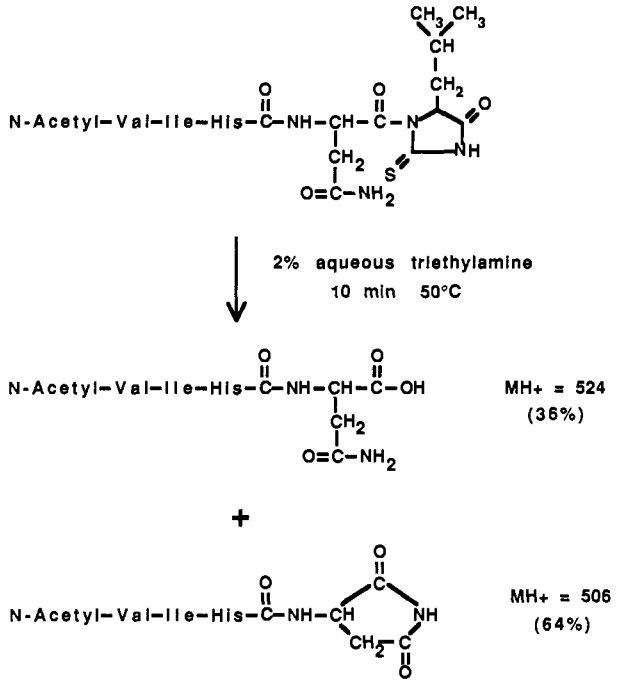

FIGURE 11: Reaction scheme for the hydrolysis of *N*-acetyl-Val-Ile-His-Asn-thiohydantoin-Leu with 2% aqueous triethylamine.

reagent (TMS-ITC) markedly improved the yields of peptidylthiohydantoin formation, allowing the use of milder reaction conditions for the derivatization of the C-terminal amino acid. This study continues this work by utilizing a model peptide, Thr-Val-Leu, to examine conditions for formation of the peptidylthiohydantoin and for hydrolysis of these peptidylthiohydantoins into an amino acid thiohydantoin derivative and a new shortened peptide capable of continued degradation. The application of this chemistry to model peptides containing many different C-terminal amino acids was also examined in order to identify any problematic residues.

Formation of Multiple Peptidylthiohydantoin Derivatives. Analysis by HPLC and FAB-MS of the products formed when schizophrenia-related peptide (Thr-Val-Leu) was reacted with TMS-ITC revealed that several peptidylthiohydantoin derivatives are formed. Besides the expected derivatives differing in acetylation at the threonine side chain, an additional derivative differing in retention time was found for each peptidylthiohydantoin. Both of these derivatives were found to have the same parent mass and identical fragmentation ions, which differed only in their relative ratios. These results suggest that these peptidylthiohydantoin derivatives with identical parent ions (in this case, two MH^+ 415 peaks and two MH^+ 457 peaks) may be optical isomers of one another. Two identical mass peptidylthiohydantoin derivatives, although in different ratios, were observed to form regardless of the C-terminal amino acid reacted except in the case when glycine was at the C-terminus. Racemization of amino acids under the conditions used for synthesis of thiohydantoins was originally observed by Csonka and Nicolet (1933). If the C-terminal amino acid on an optically active peptide was racemized during the conversion to a thiohydantoin derivative, two potentially resolvable diastereomeric peptidylthiohydantoins would be anticipated. Subsequent hydrolysis of each peptidylthiohydantoin would be expected to generate the same shortened peptide and two enantiomeric thiohydantoin amino acids which would not be resolvable. Since cleavage of these different peptidylthiohydantoins by either acetohydroxamate or aqueous triethylamine results in the same distribution of products, this double derivative formation should not be a problem in sequential C-terminal degradation, although it is of theoretical interest in understanding the mechanism of peptidylthiohydantoin formation. The fact that base catalysis by triethylamine or pyridine results in different ratios of these two identical mass derivatives to one another may also shed light on the mechanism of thiohydantoin formation. Since catalysis by triethylamine does not change the ratio of these two peaks and pyridine catalysis markedly reverses them, these two catalysts must be acting in different ways. It is likely that triethylamine acts only as a general base and catalyzes thiohydantoin formation by abstraction of a proton from the attacking peptide amide nitrogen, making it more nucleophilic, where as pyridine may catalyze the reaction by also forming a complex with the carbon of the linear acyl isothiocyanate intermediate, making the isothiocyanate carbon more electrophilic and thus more susceptible to attack by the peptide amide nitrogen. Such a complex was postulated to occur under these conditions by Miller et al. (1989) and would be analogous to that proposed by Tarr (1986) to explain tertiary amine catalysis during the N-terminal Edman degradation. Steric hindrance of neighboring amino acid side chains with such a pyridine complex may favor the formation of one isomer over the other.

Cleavage of Peptidylthiohydantoins with Acetohydroxamate. Cleavage of the peptidylthiohydantoin derivatives with acetohydroxamate as originally reported by Stark (1968) was found here to result in the formation of stable hydroxamate esters at the C-terminus of the shortened peptide. Depending on the conditions employed in this study between 68% and 93% of the peptide was derivatized at the C-terminus and thus prevented from further sequencing. Although Stark (1968) predicted such hydroxamate esters to form as an intermediate during cleavage, it was assumed that they would break down under the conditions used for cleavage or continued sequencing. This study establishes that these peptidyl hydroxamate esters, like the hydroxamate esters studied by Stieglitz and Leech (1914) and Scott and Mote (1927), are stable under the acidic conditions used for thiohydantoin formation and can only be hydrolyzed to a free peptidyl carboxylic group, capable of continued sequencing, under strongly basic conditions. This probably explains the low repetitive yields obtained by Stark (1968), Meuth et al. (1982), and Miller et al. (1989) when aqueous acetohydroxamate was employed as a cleavage reagent.

Cleavage of peptidylthiohydantoin was also attempted under aqueous acidic conditions. Incubation of peptidylthiohydantoin with 0.1 M methanesulfonic acid at 50 °C for 1 h resulted in the release of no leucine thiohydantoin. Only the uncleaved peptidylthiohydantoin was found. Methanesulfonic acid was chosen since it is equivalent to the acidic group on the resin employed by Yamashita (1971) and by Yamashita and Ishikawa (1971). It was hoped that by using the free acid any drawback associated with the eventual application of the resin-based cleavage to an automated system could be avoided. Since cleavage of protein thiohydantoins with higher concentrations of methanesulfonic acid and with the cation-exchange resin used by Yamashita (1971) was also reported by Dwulet (1976) to be unsuccessful, this method was not pursued further.

Cleavage of Peptidylthiohydantoins with Aqueous Triethylamine. Cleavage of peptidylthiohydantoins by aqueous triethylamine was originally reported by Kubo et al. (1971), Dwulet and Gurd (1979), and Meuth et al. (1982), who commented on the usefulness of triethylamine as a cleavage reagent for automated sequencing because of its volatility but declined to pursue this method, apparently in favor of cleavage

by acetohydroxamate. Cleavage of peptidylthiohydantoins by a 2% aqueous solution of triethylamine in this study was found to be rapid (half-times of 1 and 5 min at 37 and 22 °C, respectively) and quantitative, yielding only shortened peptide capable of continued sequencing and the amino acid thiohydantoin derivative. Leucine thiohydantoin, in this case, was found to be stable to the cleavage conditions even down to the subnanomole level.

Generality of Formation and Hydrolysis of Peptidylthiohydantoins. The reaction of TMS-ITC with the C-terminal amino acid of several different peptides was tested under the same general reaction conditions established with the model peptide, Thr-Val-Leu. Consideration of the side-chain functional groups leads one to expect that C-terminal Val, Phe, Gly, Trp, Ile, Ala, Met, and *S*-methyl-Cys should react similarly to the model peptide with C-terminal Leu. As expected, other peptides containing C-terminal Leu and peptides containing C-terminal Gly, Trp, Phe, and Val reacted smoothly with TMS-ITC and could be quantitatively hydrolyzed to a shortened peptide and an amino acid thiohydantoin derivative with aqueous triethylamine. Peptides with C-terminal Gln, Lys, Arg, Ser, and His behaved similarly. Problems were encountered when the C-terminal amino acids were Glu, Asp, Thr, Asn, or Pro. Problems might be expected with Glu and Asp as these amino acid side chains possess a second carboxyl group. Both glutamate and aspartate are known to form cyclic anhydrides that are not reactive to thiocyanate on reaction with acetic anhydride (Nicolet, 1930; Swan, 1952; Barker, 1953; Stark, 1968). In the case of glutamate, when the reaction with TMS-ITC was carried out in the presence of acetic anhydride, an increase in the concentration of TMS-ITC was found to produce more peptidylthiohydantoin. Also, separation of activation from coupling by first a short reaction with acetic anhydride, followed by removal of the acetic anhydride and then addition of TMS-ITC, resulted in a substantially better yield of peptidylthiohydantoin. These results suggest that when peptides containing C-terminal glutamate are reacted with acetic anhydride, formation of the five-membered oxazolinone ring, which is capable of reaction with TMS-ITC to form thiohydantoins, is initially favored. Longer exposure to acetic anhydride appears to form the more thermodynamically stable six-membered cyclic anhydride, which does not react with TMS-ITC. Therefore, a minimal reaction time with the activating reagent, acetic anhydride, is necessary to sequence through glutamate in high yield with the thiocyanate chemistry. With C-terminal aspartate, formation of the five-membered cyclic anhydride on exposure to acetic anhydride is so rapid that no formation of peptidylthiohydantoin was observed. Opening of aspartate cyclic anhydride with amine nucleophiles has been accomplished with aniline (Swan, 1952) and of peptidyl aspartate cyclic anhydrides in this study with *N*,*N*-dimethylethylenediamine and glycine ethyl ester. Although both reagents in this study were equally effective in opening up the anhydrides, peptidylthiohydantoins could only be formed from the glycine ethyl ester peptides. The peptides reacted with *N*,*N*-dimethylethylenediamine yielded only succinimide derivatives on reaction with TMS-ITC and acetic anhydride. Such a result suggests that only reagents containing a electron-withdrawing group in proximity to the nucleophilic amine, such as glycine ethyl ester and aniline, can be used to form thiohydantoins from C-terminal aspartate.

The action of acetic anhydride on threonine is known to produce an unsaturated oxazolinone which does not react with ammonium thiocyanate (Johnson & Scott, 1913; Carter, 1946). The first step in this reaction is the formation of the saturated oxazolinone. This oxazolinone can rapidly lose its active α-hydrogen to produce an unsaturated oxazolinone (Carter, 1946). In the case of C-terminal threonine, the formation of peptidyloxazolinone which can either react with TMS-ITC to form a peptidylthiohydantoin or dehydrate to form a C-terminal dehydrothreonine is consistent with the observed data. Such a dehydration was not observed to occur with C-terminal serine. It is probable that the methyl group on the threonine side chain may facilitate dehydration. Again, as with C-terminal glutamate, a limited exposure to acetic anhydride, followed by rapid removal and reaction with TMS-ITC, allows a high-yield degradation of C-terminal threonine residues. It is interesting that once the C-terminal threonine is dehydrated, it is incapable of reaction with TMS-ITC. A possible explanation is that the dehydrated C-terminal threonine can undergo a rapid conversion to a more stable imine tautomer (Figure 12) which has a more stable conjugated electron system. Such a molecule may not react with acetic anhydride to form an oxazolinone, or if an acyl isothiocyanate could form, the resulting tertiary amino group may not be nucleophilic enough for ring closure to the thiohydantoin. It is also possible that reaction of acetic anhydride with the C-terminal dehydrothreonine may form an unsaturated oxazolinone which is not capable of reaction with TMS-ITC.

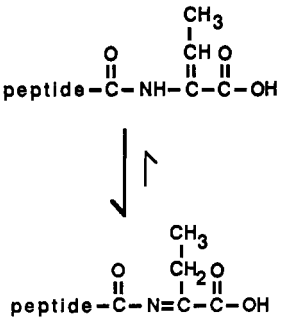

FIGURE 12: Dehydration of C-terminal threonine.

All problems with asparagine occur during hydrolysis of the peptidylthiohydantoins and are caused by five-membered cyclic imide formation with the asparagine side chain under these basic conditions. Such cyclic imide formation can occur when asparagine is in both the C-terminal and C-1 positions. When asparagine is in the C-terminal position, some of the new shortened peptide is derivatized to a C-terminal amide (31%) and is therefore incapable of continued degradation, and when asparagine is in the C-1 position, some of the new shortened peptide forms a succinimide derivative (64%) which is also not capable of continued degradation. Glutamine, which would be expected to form a six-membered cyclic imide in theory, has been found here to degrade smoothly without any evidence of cyclic imide formation.

C-Terminal proline in this study was found to be unreactive toward acetic anhydride and TMS-ITC. Proline due to its tertiary amino group has been proposed to be incapable of forming an oxazolinone (Matsuo et al., 1966; Holcomb et al., 1968), so any reaction with TMS-ITC would have to be through a linear mixed anhydride intermediate. As C-terminal proline can be activated with acetic anhydride and derivatized with glycine ethyl ester, it is possible that in the reaction with TMS-ITC a peptide isothiocyanate might be able to form as well and that the problem with proline lies in its inability to cyclize to a thiohydantoin. Elucidation of the proline problem may require a more detailed understanding of the mechanism of thiohydantoin formation. To date, the mechanism of peptidylthiohydantoin formation by acetic anhydride and TMS-ITC is not well understood (Miller et al., 1989). The racemization of the C-terminal amino acids observed in this

study suggests that reaction with acetic anhydride is forming the peptide oxazolinone (Figure 1). The formation of oxazolinones is well-known to cause racemization of amino acids (Csonka & Nicolet, 1933; Goodman & Levine, 1964). The direct reaction of the TMS-ITC molecule with the peptide oxazolinone (or peptide anhydride) was postulated by Miller et al. (1989) to be unlikely. Rather, an acetylisothiocyanate complex was predicted to form, which may then react with equilibrium amounts of protein carboxylates. Although acetyl isothiocyanate complexes may be forming in the reaction performed in the presence of acetic anhydride and TMS-ITC, work performed in this study demonstrates that the TMS-ITC molecule in acetonitrile can efficiently react with the activated peptide to form peptidylthiohydantoins. As pointed out by Stark (1968), cyclization to form a peptidylthiohydantoin with proline would require quaternization of the imino nitrogen. If our understanding of the mechanism is correct, then the lack of reactivity with proline may be caused by insufficient nucleophilic character of the tertiary amine or perhaps by steric hindrance of cyclization to the five-membered thiohydantoin ring by the proline side chain. The reaction of ammonium thiocyanate or TMS-ITC with the free amino acid proline to form proline thiohydantoin was also unsuccessful. In a recent report Inglis et al. (1989) claim to have synthesized proline thiohydantoin, although no supportive characterization data are presented.

The combination of trimethylsilyl isothiocyanate for the synthesis of the peptidylthiohydantoins and dilute aqueous triethylamine for cleavage of the derivatized peptides should now allow the thiocyanate chemistry to be applied to the sequential analysis of peptides and proteins from the carboxy terminus. The reaction conditions described here appear to be general for the high-yield formation of peptidylthiohydantoins and quantitative hydrolysis to the C-1 shortened peptide and amino acid thiohydantoin derivative for all of the C-terminal amino acids tested except for Pro, Asp, and Asn. Work in our laboratory is now directed toward improving the yields when Asp and Asn are encountered during sequential degradation and toward a solution to the proline problem. Additional work is in progress toward a solid-phase application of this method that includes automation. The solution studies reported here are not appropriate for extended C-terminal degradation since the shortened peptide must be isolated at each step.

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