NIH, for the amino acid analysis and Diana S. Parker for the end-group analyses.

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Activation of Spin-Labeled Chicken Pepsinogen†

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ABSTRACT: Chicken pepsinogen has been spin-labeled by the attachment of four nitroxides to ϵ-amino groups near the protein's amino terminus. Acidification results in a bond cleavage, generating a nonlabeled, enzymatically active protein. Electron spin resonance spectra of the spin-labeled zymogen, acidified in the presence or absence of pepstatin, are identical and indicate that the nitroxides are quite mobile, compared to the nonacidified zymogen. This mobilization is interpreted as the freeing of the peptide to which the spin-labels are attached, from the protein, subsequent to the acidification that causes a peptide bond cleavage. The rate at which the peptide leaves the protein is 1 order of magnitude slower than the cleavage of the peptide bond, measured by the rate of appearance of milk-clotting activity (first-order rate constants of 0.3 min^{-1} vs. 6 min^{-1} at pH 2, 22 °C). The inclusion of pepstatin, at molar ratios above 2 during activation, decreases the rate of peptide leaving. These observations, and those previously reported for activation of spin-labeled pig pepsinogen, are incorporated into a model of pepsinogen activation.

Conversion of pepsinogen into the active enzyme, pepsin, releases a total of about 44 amino acid residues in the activation segment (depending on the species). However, it has been shown (Dykes & Kay, 1976; Christensen et al., 1977) that these amino acids are not all released together as one peptide—instead a sequential activation mechanism is operative. Of the zymogens of this type, pig pepsinogen has been studied most extensively. By inclusion of a small molar excess of pepstatin to arrest the activation at completion of the first step, it was found that the first peptide bond to be hydrolyzed is Leu_{16}–Ile_{17} (Figure 1). A peptide of 16 amino acid residues is released, generating an intermediate, "pseudopepsin" (Kay & Dykes, 1977). Activations of chicken pepsinogen and calf prochymosin also occur by sequential mechanisms (Kay & Dykes, 1977; Keilova et al., 1977; Pedersen et al., 1979), but in both these cases the first bond split is Phe_{25}–Leu_{26} (Figure 1) and a larger initial peptide is released. Activation in the presence of pepstatin is restricted to cleavage of the Phe–Leu

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                1               5                  10
Pig          Leu-Val-Lys-Val-Pro-Leu-Val-Arg-Lys-Lys-Ser-Leu-Arg-Gln-

Chicken   Ser-Ile-His-Arg-Val-Pro-Leu-Leu-Lys-Gly-Lys-Ser-Leu-Arg-Lys-

               15                 20                 25
Pig       Asn-Leu/Ile-Lys-Asp-Gly-Lys-Leu-Lys-Asp-Phe-Leu-

Chicken   Gln-Leu-Lys-Asp-His-Gly-Leu-Leu-Glu-Asp-Phe/Leu-
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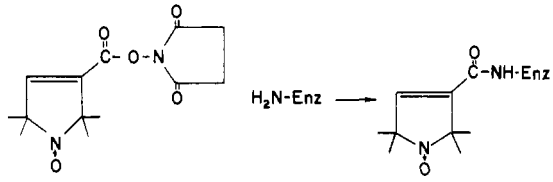
FIGURE 1: Amino-terminal sequences of pig and chicken pepsinogens according to Kostka et al. (1981). A slant (/) indicates the first bond cleaved during activation.

bond with no possibility of further conversion of the inactivation pepstatin–pseudopepsin complex.

The investigation of molecular changes within protein molecules has been greatly facilitated by the introduction of spin-labeling reagents, and in a previous report it was shown that pig pepsinogen reacted with such a reagent in the amino-terminal region of the activation segment (Twining et al., 1981) with a reagent designed to label the amino groups of an enzyme or other protein. By following changes in the



electron spin resonance (ESR) spectra on acidification of spin-labeled pepsinogen to pH 2, it was shown that the rate of mobilization of the spin-label (i.e., liberation of the 1–16 peptide) was about 1 order of magnitude slower than the rate of generation of an alkali-labile derivative (i.e., the pseudopepsin formed by hydrolysis of the bond between residues 16 and 17). This discrepancy is most simply explained if the 16-residue peptide remained bound transiently and dissociated only slowly from the pseudopepsin molecule after scission of the bond between residues 16 and 17. Indeed, it has been shown that this peptide binds readily to pig (and to ox) pepsin(s) (Harboe et al., 1974; Harish-Kumar & Kassell, 1977; Dunn et al., 1978) but not to calf chymosin (Kay & Dykes, 1977) nor to chicken pepsin.

In an attempt to test this interpretation, we examined the kinetics of activation of spin-labeled chicken pepsinogen. The zymogen from this species is particularly appropriate for use in these studies since we had shown previously (Keilova et al., 1977) that the (somewhat larger) 25-residue peptide released in the first step in the activation of this protein does not bind significantly to chicken (nor to pig) pepsin.

Experimental Procedures

Materials. Chicken pepsinogen was prepared according to Kostka et al. (1977). 3-[[(2,5-Dioxo-1-pyrrolidinyl)oxy]-carbonyl]-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrolyl-1-oxy (Chemical Abstracts Service Registry No. 37558-29-5) was purchased from Molecular Probes (Plano, TX); pepstatin was from Sigma (Poole, Dorset, U.K.). Keynote dried skim milk powder was purchased from Littlewoods (Newport, U.K.).

General Methods. The milk-clotting activity of pepsin at pH 5.3 was assayed according to McPhie (1976). Chicken pepsinogen and spin-labeled chicken pepsinogen were reduced and S-sulfonated as described by Pechere et al. (1958). Determination of amino-terminal residues in the native and reduced/modified proteins was by the dansylation method of Gray (1967).

Spin-Labeling of Pepsinogen. A solution of 3.4 mg of 3-[[(2,5-dioxo-1-pyrrolidinyl)oxy]carbonyl]-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrolyl-1-oxy in 0.08 mL of dimethylformamide was added to chicken pepsinogen (15 mg) in 2.7 mL of 0.1 M sodium phosphate buffer, pH 7.2. This solution was agitated at 22 °C for 8 h and then dialyzed against several changes of a suspension of activated charcoal powder in 0.01 M sodium phosphate buffer, pH 7, at 4 °C. The concentration of nitroxide in the solution of pepsinogen was determined as described previously (Twining et al., 1981). ESR spectra were measured in 50-μL capillary tubes on a Varian E-109 spectrometer with 100-kHz modulation and which had a V4540 temperature controller attached. The spectra were read at 21 °C. Chicken pepsinogen concentration was calculated from $\epsilon_{280} = 5.0 \times 10^4$ L mol^{-1} cm^{-1} (Bohak, 1969).

Rate of Activation of Native and Spin-Labeled Pepsinogens. The activation of pig pepsinogen is followed conventionally by removing aliquots at appropriate times into alkaline buffer, pH 8.5, to stop the activation process and to denature the pig pepsin generated (Al-Janabi et al., 1971; Twining et al., 1981). Reacidification to pH 2 activates the residual pepsinogen, and the proteolytic activity thus generated is measured by using hemoglobin as the substrate. This method is inappropriate, however, for use with chicken pepsinogen since chicken pepsin is stable up to pH 9.0 (Bohak, 1973). Thus, instead of following the rate of disappearance of chicken pepsinogen, we measured the rate of generation of milk-clotting activity directly for this system. Aliquots of acidified chicken pepsinogen were quenched at pH 8.5, and the activity generated was measured in the milk-clotting assay at pH 5.3. No further activation of chicken pepsinogen took place under these conditions. In a typical experiment, chicken pepsinogen (0.25 mg in 0.25 mL containing 1.6 μmol of phosphate and 4.5 μmol of borate at pH 10.3) was acidified with 10–40 μL of 0.1 M HCl at 22 °C. At times from 6 to 150 s, aliquots (50 μL) were removed into 0.20 mL of 0.05 M sodium borate buffer, pH 8.5, at 0 °C. These quenched solutions were assayed as described by McPhie (1976). Semilog plots of reciprocal clotting time vs. activation time gave first-order rate constants for the activation.

Kinetics of ESR Spectral Changes. Spin-labeled chicken pepsinogen (200 μL) that had been dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, was acidified with varying amounts of 0.3 M HCl and immediately placed in sealed capillary tubes, and the high-field ESR peak was repeatedly scanned to detect changes in peak intensity.

Results

The ESR spectra of spin-labeled chicken pepsinogen at pH 7 and at pH 2 are shown in Figure 2. Comparison of the protein with the nitroxide concentrations indicates that 3.72 nitroxides were bound per mol of protein. Some characteristics of the spectra are presented in Table I. Of particular interest

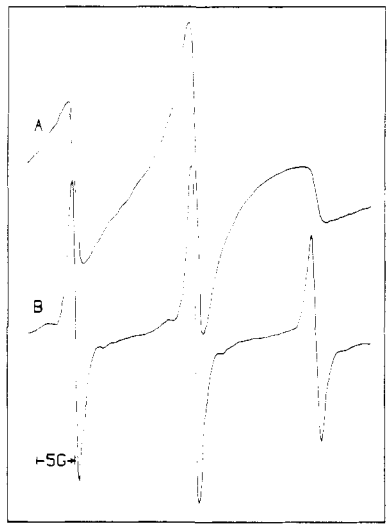


FIGURE 2: ESR spectra of spin-labeled chicken pepsinogen and an activated form. (A) Spin-labeled chicken pepsinogen (35 μM) in 0.01 M sodium phosphate buffer, pH 7. (B) Same solution as (A) brought to pH 2 with HCl. Conditions: 100-kHz modulation; 20-mW microwave power (9.51 GHz); modulation amplitude 2 G; scan range 100 G; time constant 0.128 s.

Table I: Characteristics of Electron Spin Resonance Spectra[a]

sample	peak intensity ratios h_1/h_0	h_0/h_{-1}	τ (ns)
spin-labeled pepsinogen, pH 7	0.57	5.39	2.1
spin-labeled pepsinogen, pH 2	0.90	1.61	0.3
spin-labeled pepsinogen, pepstatin, pH 7	0.53	5.49	2.3
spin-labeled pepsinogen, pepstatin, pH 2	0.91	1.38	0.2
spin-labeling reagent	0.99	1.09	0.05

[a] The signal peak intensities were measured in the ESR spectra from Figure 3 and from other spectra that are not shown. The correlation time, τ, was calculated according to Kivelson (1960) by taking the mean of values obtained from linear and quadratic terms.

are the peak intensity ratios, which are descriptive of each spectrum and which, by their variance from unity, indicate the degree of immobility of the nitroxides. Acidification of the spin-labeled chicken pepsinogen appeared to mobilize the nitroxides to the same extent that acidification affected spin-labeled pig pepsinogen, where the correlation constant changed from 1.55 to 0.13 ns (Twining et al., 1981). In that case the labeled peptide that was generated was chromatographically separated from the pepsin, so the mobilization of nitroxides could be unambiguously attributed to the separation of the labeled peptide from the protein. While we did not chromatographically separate the peptide from the enzyme in this study, we have assumed that the behavior of the two labeled zymogens is identical in this respect. (There are no reports of the instability of nitroxides at the pH values we employ.) In the pH 2 spectrum of spin-labeled chicken pepsinogen (Figure 2) there remains no indication of any residual immobilized spin-label, which, by analogy to the case of spin-labeled pig pepsinogen and in its own right, strongly suggests that the reason the resultant protein has no nitroxide attached to it is that the labeled peptide has been freed from the macromolecule.

The ESR spectra measured for pepsinogen in the presence of pepstatin (molar ratio 1:3.4) were identical with those obtained in the absence of pepstatin (Table I). This is unremarkable at pH 7 since pepstatin does not interact with pepsinogen at neutral pH (Kay & Dykes, 1976), but the identity of the spectra at pH 2 indicates that all four nitroxides bound to pepsinogen must have been released on acidification of the spin-labeled pepsinogen. As activation in the presence of pepstatin is restricted (Keilova et al., 1977) to the liberation of only one peptide (residues 1–25, Figure 1), it appears that all four nitroxides must have been attached within the 25-residue peptide.

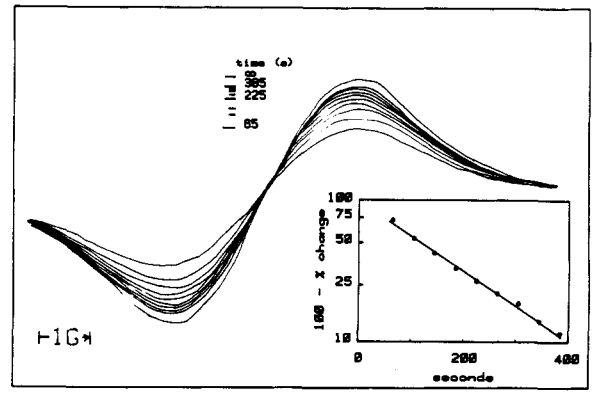


FIGURE 3: Repeated scans of the high-field peak following activation. The ESR spectrum of the peak was scanned at 40-s intervals following acidification of 2.20 mL of a 34 μM solution of spin-labeled pepsinogen with 0.02 mL of 0.3 M HCl (final pH 2.25). Inset shows a semilog plot of the data vs. time.

Since the spin-labeling reagent is designed to react with amino or other nucleophilic groups, the potential sites of reaction in the activation peptide are (a) the α-amino group of the amino-terminal serine, (b) the ϵ-amino groups of the four lysine residues in positions 8, 10, 14, and 17, and, possibly, (c) the two serine residues at the amino terminus and position 11 (Figure 1). Native chicken pepsinogen and spin-labeled chicken pepsinogen were reacted with dansyl chloride, but after acid hydrolysis and thin-layer chromatography on polyamide sheets, it was not possible to detect Dns-serine in either case. In keeping with previous observations, this suggests that the α-amino group is buried in this protein. Samples of the native and spin-labeled proteins were denatured by reduction and S-sulfonation and then reacted with dansyl chloride. After acid hydrolysis, spots of Dns-serine were observed (in approximately equivalent amounts) for the unmodified and spin-labeled proteins. Quantitation of the amounts of ϵ-Dns-lysine was not possible because of the similarity of R_f values of this derivative to that of the (excess of) dansyl hydroxide, which is always observed on the polyamide sheets. Thus it would appear that the amino-terminal serine residue is still free in the spin-labeled chicken pepsinogen. Since this residue is apparently not available in the native protein at pH 7–8 to an amino group modifying reagent (dansyl chloride), it is perhaps not surprising that another, bulkier, amino group reagent (the spin-labeling reagent) failed to gain access to the amino terminus. By difference, then, it would appear that this reagent had reacted only with the side chains of four lysine residues, probably those in positions 8, 10, 14, and 17. However, since we did not directly determine the sites of spin-labeling and since the *N*-hydroxysuccinimide ester reagent may conceivably modify serine hydroxyls, our conclusion that the four lysines are the sites of modification must bear this qualification.

The kinetics of the acid-initiated transformation of the spectrum of Figure 2A to that of Figure 2B was studied by focusing on that part of the spectrum that shows the greatest change during activation, namely, the high-field peak. During

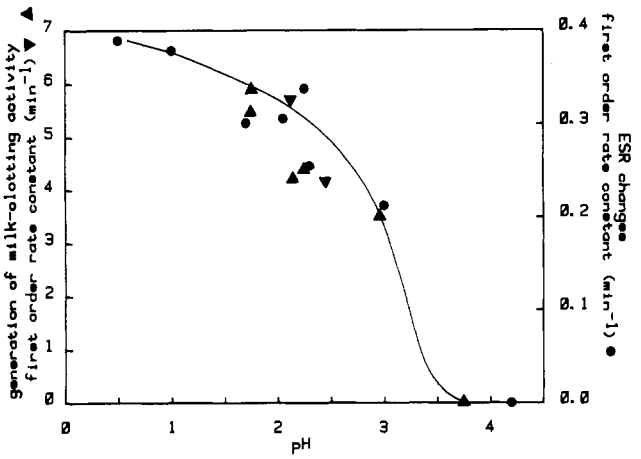


FIGURE 4: Rate constants vs. pH at 22 °C. (▼) Conversion of chicken pepsinogen to an enzymatically active form. (▲) Conversion of spin-labeled chicken pepsinogen to an enzymatically active form. (●) Change in the ESR signal of spin-labeled chicken pepsinogen.

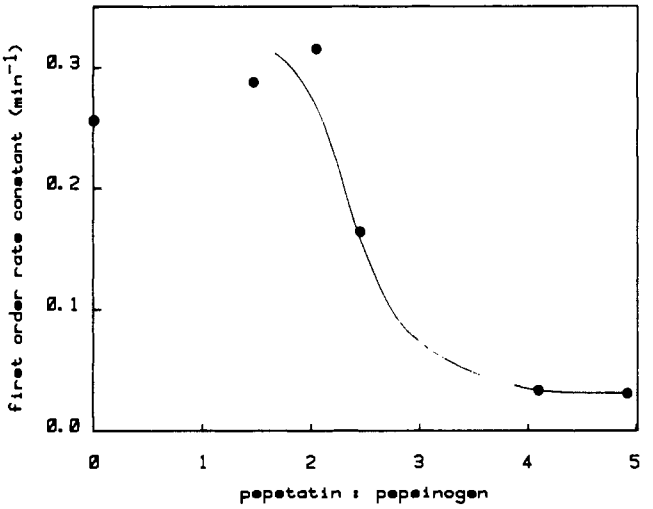


FIGURE 5: Rate of change at pH 2.7 of the ESR signal of spin-labeled chicken pepsinogen vs. pepstatin concentration. The point for the rate in the absence of pepstatin is interpolated from the data in Figure 4. The concentration of spin-labeled pepsinogen in these experiments was between 24 and 28 μM.

activation h_{-1} increased (Figure 3). This was followed with time, and first-order rate constants were extracted from the semilog plots (e.g., Figure 3 inset). Similar experiments were performed over a pH range from 0.5 to 4.2, and the rate constants are presented in Figure 4. These are compared with the rate constants for activation as measured by the generation of milk-clotting activity from acidified zymogen (Figure 4).

The effect of altering the pepstatin concentration on the rate of release of the activation peptide from spin-labeled chicken pepsinogen is shown in Figure 5. At ratios of pepstatin to pepsinogen above 2:1, there was a very pronounced lowering of the rate of release of the peptide. This is very similar to the earlier observation (Dykes, 1978) that increasing the molar ratio of pepstatin to pepsinogen decreased the yield of the first peptide released during the "stopped" activation of the zymogen.

Discussion

As was found previously with spin-labeled pig pepsinogen (Twining et al., 1981), spin-labeled chicken pepsinogen is a faithful model for the native zymogen in activation studies: an active enzyme is generated (that does not contain spin-label), and the rates of activation of unlabeled and labeled zymogens are very similar, as are the pH dependencies of the rates of activation.

The slower release of the spin-labeled activation peptide compared to the faster commitment to peptide bond cleavage that was observed previously with pig pepsinogen (Twining et al., 1981) was found also in this study with spin-labeled chicken pepsinogen. Inclusion of a small molar excess of pepstatin during the activation of pig pepsinogen resulted in the rate of leaving of the 1–16 activation peptide being increased to a value similar to that measured for peptide bond cleavage. This effect was not observed, however, with the chicken pepsinogen system. A model to account for both these findings with pig and chicken pepsinogen activation is presented in Figure 6.

The effect of higher concentrations of pepstatin on the rate of leaving of the activation peptide (Figure 5) can best be explained by postulating that pepsinogen (I) is in equilibrium with a conformation that has a partially formed substrate binding site (II) that can bind pepstatin reversibly (IIa). When unoccupied with pepstatin, the binding site can accommodate that section of the activation peptide that contains the scissle peptide bond (III). At the right pH, that bond is cleaved (IV). The resulting peptide is not held to the protein by its carboxyl-terminal residues—it has already been shown that residues 13–16 can be removed from the pig peptide without affecting its ability to inhibit pig pepsin (Dunn et al., 1978)—so the points of contact must be further distant from the carboxyl terminus of the peptide and, at the instant of scission, distant from the catalytic subsites (V). Inhibition by the activation

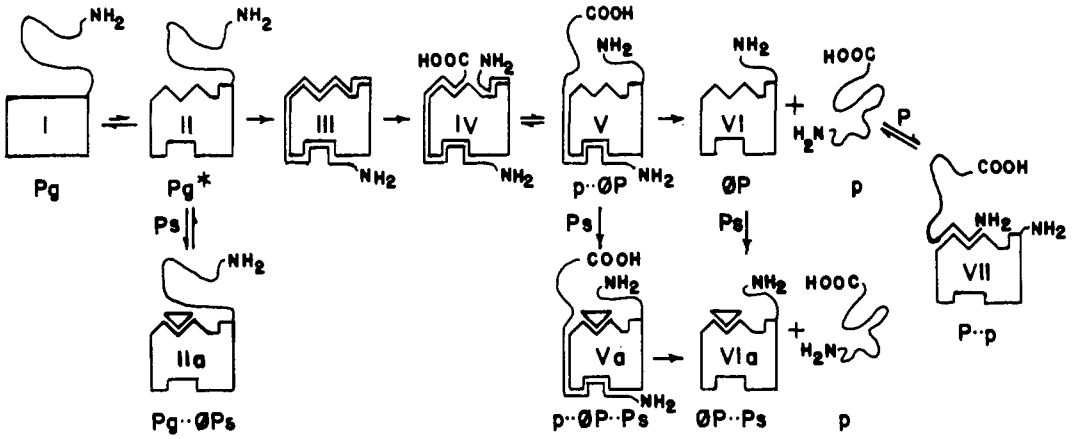


FIGURE 6: Model for activation of pepsinogen. Abbreviations: Pg, pepsinogen; ΦP, pseudopepsin; P, pepsin; Ps, pepstatin; p, peptide 1–16. A simpler model would not distinguish between I and II, but by making this distinction, we follow Marciniszyn et al. (1976b).

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†

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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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