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Irreversible Degradation of Histidine-96 of Prothrombin Fragment 1 during Protein Acetylation: Another Unusually Reactive Site in the Kringle†

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ABSTRACT: Acetylation of prothrombin fragment 1 in acetate–borate buffer at pH 8.5 resulted in the appearance of increased light absorbance at about 250 nm. Protease digestions resulted in isolation of a single peptide (residues 94–99) with intense absorbance at about 250 nm (estimated extinction coefficient of 5000 M^{-1} cm^{-1}). Amino acid analysis showed the expected composition except for the absence of His-96. Instead, an unidentified amino acid which had a ninhydrin product with absorption properties similar to those of proline eluted near aspartate. When sequenced, this peptide (YP?KPE containing ε-amino-acetyllysine) lacked histidine at the third position but gave a high yield of a PTH derivative that eluted near PTH-Gly from the HPLC column. Fast atom bombardment mass spectrometry of the derivatized 94–99 peptide showed a mass that was 74 units higher than expected. The histidine degradation product was identified as a di-N-acetylated side chain with an opened imidazole ring and loss of C_2 of the ring. While a similar degradation pattern has previously been reported during acylation of histidine, the high chemical reactivity exhibited by His-96 was unusual. For example, under conditions sufficient for quantitative derivatization of His-96, His-105 of fragment 1 was not derivatized to a detectable level. Furthermore, His-96 in fragment 1 was at least an order of magnitude more susceptible to degradation than His-96 in the isolated 94–99 peptide. His-96 is therefore one of several neighboring amino acids of the kringle portion of fragment 1 that displays highly unusual chemistry (see also Asn-101 [Welsch, D. J., & Nelsestuen, G. L. (1988) *Biochemistry 27* 4946–4952] and Lys-97 [Pollock, J. S., Zapata, G. A., Weber, D. J., Berkowitz, P., Deerfield, D. W., II, Olson, D. L., Koehler, K. A., Pedersen, L. G., & Hiskey, R. G. (1988) in *Current Advances in Vitamin K Research* (Suttie, J. W., Ed.) pp 325–334, Elsevier Science, New York]). Unusual ^1H NMR signals from histidine residues in the analogous position of other kringle sequences have been reported as well [Hochswender, S. M., Laursen, R. A., De Marco, A., & Llinas, M. (1983) *Arch. Biochem. Biophys. 223*, 58–67; Llinas, M., De Marco, A., Hochschwender, S. M., & Laursen, R. A. (1983) *Eur. J. Biochem. 135*, 379–391; Trexler, M., Banyai, L., Patthy, L., Pluck, N. D., & Williams, R. J. P. (1983) *FEBS Lett. 154*, 311–318]. This region of kringle structures may constitute an unusual component determined by folding of the kringle.

Prothrombin is a vitamin K dependent protein which is required in the penultimate step of the coagulation cascade (Stenflo & Suttie, 1977; Nemerson & Furie, 1980; Nelsestuen, 1984). It requires a substantial amount of posttranslational modification for function [i.e., cleavage of the pre- and propeptides (Degen et al., 1983; MacGillivray & Davie, 1984), glycosylation (Magnusson et al., 1975), and the vitamin K dependent conversion of specific Glu residues to γ-carboxyglutamic acid (Gla)[1] residues (Nelsestuen et al., 1974; Stenflo et al., 1974)]. Prothrombin fragment 1 (the amino-terminal 156 amino acids of prothrombin) contains all 10 of the Gla residues of prothrombin as well as a triple-looped sequence of amino acids known as a kringle (Magnusson et al., 1975). The binding of a variety of metal ions to fragment 1 causes the peptide to undergo a conformational change that can be observed by a decrease in intrinsic protein fluorescence (Nelsestuen, 1976; Prendergast & Mann, 1977). This metal ion induced conformational change is required for subsequent protein binding to phospholipid surfaces (Nelsestuen et al., 1976). The precise nature of the metal ion and membrane

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; fragment 1, amino acids 1–156 of the amino terminus of bovine prothrombin; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin.

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binding sites on fragment 1 has been of intense interest. While the Gla residues are generally recognized as essential components for protein function, the exact nature of their involvement has yet to be determined. Furthermore, identification of other important amino acids or peptide domains has been limited.

Structure–function relationships can be investigated by chemical modification of native protein. Recent investigations have demonstrated the importance of the amino-terminal alanine for the process of membrane binding by prothrombin (Welsch & Nelsestuen, 1988a). These studies also revealed that carbohydrate-linked Asn-101 was essential to the fluorescence quenching event (Welsch & Nelsestuen, 1988b). This observation implicated the kringle region of fragment 1 as important to prothrombin function.

The current investigations were initiated to better understand the structure–function relationship of the vitamin K dependent and kringle-containing fragment 1 peptide and to identify specific amino acids that show unusual chemical properties. The results provided direct chemical evidence that His-96 was highly reactive toward acetic anhydride in intact fragment 1 and that this reactivity was unique to this histidine. This investigation, in conjunction with previous results, begins to establish that this region of kringle structures has highly unusual chemical properties. This may signify an important, although as yet, unidentified function for this region of the kringle.

Materials and Methods

Materials. Boric acid, sodium acetate, trypsin (type XIII), V8 protease from *Staphylococcus aureus* (type XVII), and the synthetic tripeptide Arg-His-Phe were purchased from Sigma (St. Louis, MO). Sephadex G-100 SF was from Pharmacia. All reagents were reagent or HPLC grade. All solutions were prepared with distilled and deionized water.

Bovine prothrombin fragment 1 was prepared as previously described (Welsch et al., 1988). The purified fragment 1 gave a single band when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970), and the intrinsic fluorescence decrease (Nelsestuen, 1976) induced by saturating amounts of calcium was at least 55%. Protein concentrations were quantitated by using an $E^{1\%}_{280nm}$ of 10.1 (Heldebrant & Mann, 1973). A molecular weight of 23 400 was used for calculations of molar concentrations.

Acetylation Conditions. Histidine-containing samples (43 μM) were acetylated at ambient temperature for 30 min with acetic anhydride in 1.0 M sodium acetate–0.25 M borate buffer, pH 8.5. The degree of acetylation was controlled by varying the molar ratio of acetic anhydride to histidine. Routinely, samples were acetylated in buffer containing 40 mM acetic anhydride. The Arg-His-Phe tripeptide was not highly reactive and was subjected to several such rounds of acetylation to obtain adequate product. The pH of the reactions was maintained at approximately 8.5 by addition of 4 M sodium hydroxide. Calcium-protected samples were prepared by preincubation of the peptide with saturating (25 mM) calcium ions in the acetate–borate buffer. These conditions gave the same degree of calcium-dependent change in intrinsic protein fluorescence as was reported for titrations in more dilute buffer (Resnick & Nelsestuen, 1980).

Before analysis of acetylated samples, labile acetyl groups were removed from samples by hydroxylamine treatment [0.2 M hydroxylamine (pH 8.0) at 25 °C for 2 h]. This reaction was usually carried out in the buffer used for acetylation.

Peptide Isolation. Chromophore-containing samples of fragment 1 (7.5 mg/mL) and Arg-His-Phe (1 mg/mL) were treated with trypsin [trypsin to peptide ratio equal to 1:30 (w/w)] in 25 mM ammonium bicarbonate, pH 7.5, for 6 h at 37 °C. The fragment 1 digest was fractionated by gel filtration on a Sephadex G-50 column (2.0 × 96.0 cm) eluted with ammonium bicarbonate buffer. Selected fractions were pooled and lyophilized.

Peptide mixtures from the gel filtration column as well as the tryptic products from the acetylated tripeptide were separated by reversed-phase HPLC using an Aquapore RP-300 column (4.6 mm × 25 cm, RAININ). This C8 column chromatography was performed on a Varian 5020 liquid chromatograph equipped with a Varian UV-50 detector and Model 9176 recorder. A constant flow rate of 1.0 mL/min was used for elution of peptides. Elution was accomplished by increasing the percentage of organic phase [acetonitrile containing 0.1% trifluoroacetic acid (TFA)] relative to the initial aqueous phase (0.1% TFA in water). The program used for elution of peptides consisted of aqueous phase (0–5 min), a linear gradient of increasing organic phase to 20% (5–25 min), increasing linear gradient to 23% (25–31 min), increasing linear gradient to 27% (31–43 min), and to 30% organic phase at 49 min. The tryptic product from the tripeptide which contained chromophore was pooled and lyophilized.

The chromophore-containing tryptic peptide from fragment 1 was further digested with V8 protease [V8 protease to peptide ratio of 1:10 (w/w)] in 100 mM sodium acetate at pH 4.0 for 8 h at 37 °C. The products were separated by HPLC (as outlined above), and the peptide-containing chromophore was pooled and lyophilized.

Other Procedures. Absorption spectra were measured with a Beckman DU-8 UV–visible spectrometer with a slit width of 0.5 nm. Samples for amino acid analysis were hydrolyzed in 6 N HCl at 110 °C for 16 h in evacuated, sealed tubes. Amino acid compositions were determined with a Beckman System 6300 high-performance analyzer. Automatic Edman degradation was carried out in an ABI Model 470 A sequenator as previously described by Hewick et al. (1981). Phenylthiohydantoins were identified by reversed-phase HPLC.

Fast atom bombardment mass spectra (FAB/MS) were obtained with a Kratos MS-25 high-resolution mass spectrometer equipped with an Ion Tech fast atom gun. FAB ionization employed 8-keV xenon atoms impinging on a copper target. The spectra were recorded with an accelerating voltage of 1.33 keV at a scan rate of 3.0 s/decade. Samples were dissolved in water (~2–10 μg/μL), and about 1 μL was applied to the target following the application of approximately 5 μL of glycerol.

^{1}H NMR spectra were recorded on an NT 300WB spectrometer operating in the Fourier-transform mode at a frequency of 300 MHz. Spectra were recorded at 25 °C. The protein samples were dissolved in D_2O (99.96 atom % deuterium; Sigma Chemical Co.). The field was locked on the deuterium signal, and the chemical shifts are reported relative to external tetramethylsilane. Spectra were obtained with an 8-μs pulse, a 4800-Hz spectral width, and a 0.85-s acquisition time.

Results

Production and Isolation of the Chromophore. Fragment 1 and the synthetic peptide Arg-His-Phe were acetylated in acetate–borate buffer at pH 8.5 as described under Materials and Methods. The samples were treated with hydroxylamine to remove acetyl groups that were incorporated at labile sites. Comparison of the absorption spectra of native fragment 1 before and after these procedures revealed increased absor-

Table I: Amino Acid Analysis

assignment	tryptic peptide[a] after HPLC	Figure 3, peptide A	Figure 3, peptide B	Figure 3, peptide C	modified synthetic peptide
Tyr	0.96[c] (1)[d]	0.73 (1)	-	0.79 (1)	-
Pro	2.73 (3)	1.80 (2)	0.88 (1)	1.83 (2)	-
His	0.88 (1)	0.08 (0)	0.91 (1)	0.87 (1)	-
Lys	1.09 (1)	1.00 (1)	-	1.00 (1)	-
Glu	1.13 (1)	1.18 (1)	-	1.14 (1)	-
Ile	0.94 (1)	-	0.96 (1)	-	-
Ser	1.01 (1)	-	0.83 (1)	-	-
Thr	1.72 (2)	-	1.79 (2)	-	-
Gly	1.02 (1)	-	1.04 (1)	-	-
Ala	1.00 (1)	-	1.00 (1)	-	-
Asp	2.03 (2)	-	1.98 (2)	-	-
Leu	1.20 (1)	-	1.14 (1)	-	-
Arg	1.16 (1)	-	1.09 (1)	-	-
Phe	-[e]	-	-	-	1.00 (1)
deg. His[b]	+[f] (1)	+ (1)	-	-	+ (1)

[a] This composition was obtained for fragment 1 which had been subjected to two rounds of acetylation (40 mM acetic anhydride each). After a single round of acetylation, the His content of the isolated peptide was 1.39. [b] "deg. His" denotes the degradation product of histidine produced by acetylation. [c] The values in the table represent the amino acid mole fractions calculated relative to the amino acid with mole fraction 1.00. [d] Predicted amino acid mole fractions are given in parentheses. [e] A dashed line (-) indicates that this amino acid was present at background levels. The background values were always ≤10% of those given. [f] A plus sign (+) indicates the presence of this component.

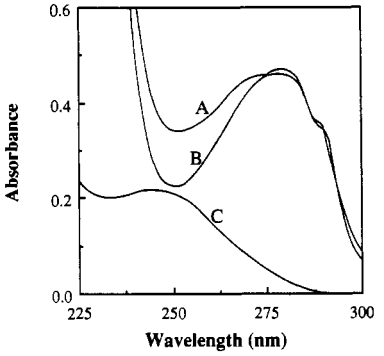

FIGURE 1: Absorption spectra of selected peptide derivatives. Shown are the absorption spectra of acetylated fragment 1 [40 mM acetic anhydride (A)] and native fragment 1 (B) in acetate–borate buffer after treatment with hydroxylamine as described under Materials and Methods. The protein concentration was 20 μM. Also shown is the absorption spectrum of the acetylated Arg-His-Phe peptide at a concentration of 40 μM.

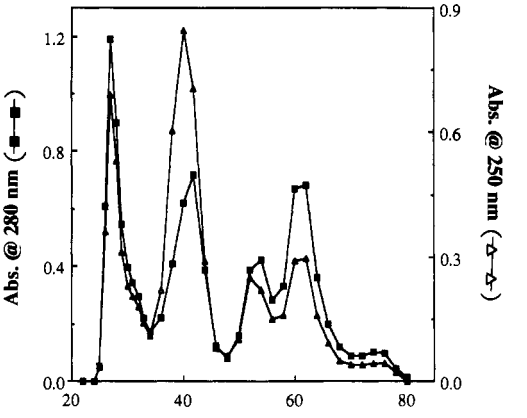

FIGURE 2: Elution profile of tryptic peptides of acetylated fragment 1. The tryptic digestion products of acetylated fragment 1 were fractionated on a Sephadex G-50 column (2.0 × 96.0 cm) equilibrated in 25 mM ammonium bicarbonate (pH 7.5). The absorbance at 280 nm (■) and 250 nm (△) of selected fractions is shown.

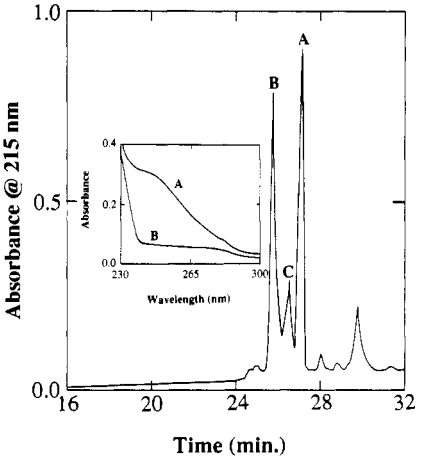

FIGURE 3: Isolation of the chromophore by HPLC. The products from V8 protease digestion of the 94–111 peptide of acetylated fragment 1 were separated by reversed-phase HPLC. This fragment 1 had been subjected to a single acetylation (40 mM acetic anhydride). The elution profiles for the 100–111 peptide (peak B) and the 94–99 peptide containing derivatized lysine and histidine (peak A) are shown. Peak C was identified as the 94–99 peptide containing derivatized lysine but normal histidine. Inset: Absorption spectra of peaks A and B.

bance at 250 nm (Figure 1A,B). Furthermore, the synthetic tripeptide yielded a product with absorption properties that could explain the absorption properties of the derivatized fragment 1 (Figure 1C).

Acetylated fragment 1 was digested with trypsin and chromatographed on Sephadex G-50 (Figure 2). Measurement of A_{250} and A_{280} revealed that fractions 35–46 contained peptides having unusually high absorbance at the shorter wavelength. Previous investigations have revealed that the carbohydrate-linked tryptic peptides of fragment 1 elute at this position (Welsch & Nelsestuen, 1988b). The production of the new chromophore was not influenced by metal ions. That is, fragment 1 derivatized in the presence of 25 mM calcium gave results similar to the apoprotein (data not shown).

Reversed-phase HPLC followed by amino acid analysis revealed that the chromophore was associated with the 94–111 carbohydrate-linked peptide (Table I, column 2). Further digestion of the 94–111 peptide with V8 protease followed by HPLC separation gave the expected digestion products (Figure 3). The absorption spectra and amino acid analysis of the resultant peptides showed that the chromophore was associated exclusively with the 94–99 peptide (Figure 3, inset, and Table I).

When fragment 1 had been treated with a single round of acetylation, amino acid analysis (Table I) of the peptides

Table II: Amino Acid Sequence Analysis

sample	sequence turn 1	2	3	4	5	6
Figure 3, peptide A	Tyr (1.23)[a]	Pro (1.33)	M. His[b] (+)[c]	AcLys[d] (+)	Pro (1.11)	Glu (0.56)
modified synthetic peptide	M. His (+)	Phe (0.68)				

[a] The values in parentheses represent the number of nanomoles of the predominant amino acid released per cycle. The amount of predominant amino acid identified was always at least 10-fold greater than the next most abundant amino acid. A total of 1.5 nmol was applied for each sample. [b] "M. His" denotes the modified product of histidine produced by acetylation. [c] A plus sign (+) indicates the presence of this component. [d] "AcLys" denotes ε-aminoacetyllysine.

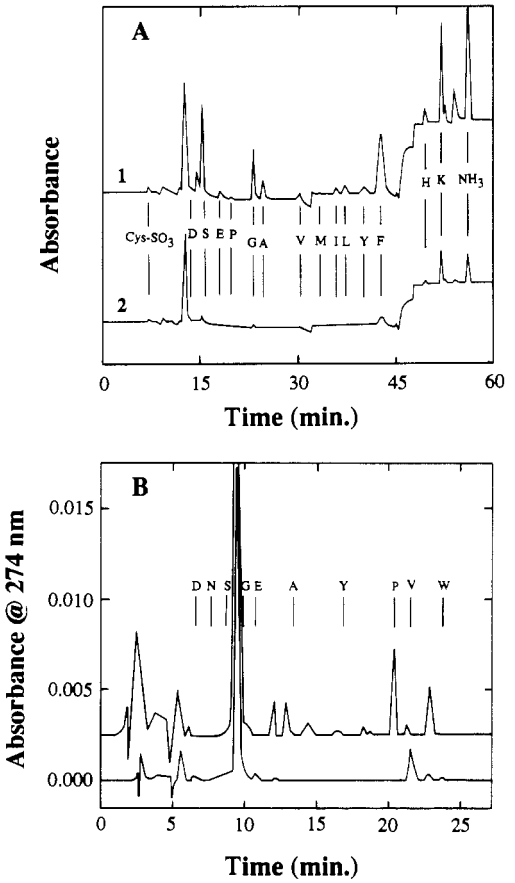

FIGURE 4: Characterization of the derivatized histidine. Panel A shows the elution profile of the amino acid analysis of the acetylated His-Phe dipeptide. The absorbance was monitored at 440 and 540 nm (scan 1) or only at 440 nm (scan 2). Panel B shows the HPLC elution profiles for the third PTH derivative released from the chromophore-containing 94–99 peptide (upper scan) and for the first PTH derivative released from the acetylated His-Phe dipeptide (lower scan). The upper scan was offset 0.0025 absorbance unit, and the peak corresponding to proline represents carryover from the previous cycle.

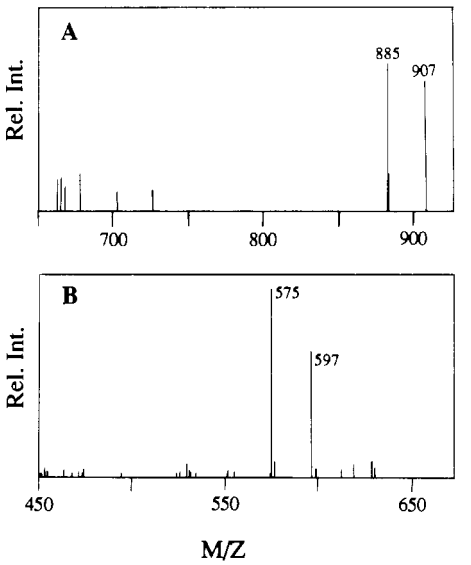

FIGURE 5: Fast atom bombardment mass spectra of chromophore-containing peptides. Shown are the FAB/MS spectra of the isolated 94–99 peptide from acetylated fragment 1 (panel A) and the acetylated Arg-His-Phe tripeptide (panel B). The primary peak corresponds to the M + 1 ion and the secondary peak corresponds to the M + Na^+ ion in both cases. These peaks should occur at 811 and 833 mass units for the peptide YPHKPE (containing acetylated lysine) and 501 and 523 mass units for the RHF tripeptide (containing acetate at the amino terminal).

produced by V8 digestion demonstrated that two forms of the 94–99 peptide were present: (1) the 94–99 peptide with acetylated Lys-97 and a modified His-96 (peptide A, Figure 3) and (2) the 94–99 peptide with acetylated Lys-97 but normal histidine (peptide C, Figure 3). On the basis of the yield at this stage of isolation, a single acetylation resulted in 40% degradation of His-96. Other experiments which produced quantitative destruction of His-96 (Table I, column 2) were conducted with the more exhaustive acetylation procedure. The spectra in Figure 3 (inset) show little or no chromophore associated with His-105 (peptide B). This property also extended to fragment 1 which had been subjected to two acetylation reactions (data not shown). Thus, His-105 appeared to be extremely unreactive.

The synthetic peptide, Arg-His-Phe, was extensively acetylated and digested with trypsin (as outlined under Materials and Methods). The resulting chromophore-containing His-Phe dipeptide was isolated by reversed-phase HPLC (data not shown).

Characterization of Isolated Chromophore-Containing Peptides. The chromophore-containing peptide isolated from acetylated fragment 1 and the chromophore associated with the acetylated His-Phe dipeptide were analyzed by amino acid analysis, sequence analysis, fast atom bombardment mass spectrometry, and ^1H NMR. Amino acid analyses gave the anticipated composition for both of these peptides except for the absence of histidine (Table I). Instead, an unidentified peak eluted near aspartic acid (Figure 4A). This unidentified peak gave a ninhydrin product which had absorption properties characteristic of proline (Figure 4A, scan 2). These results suggested that the chromophore produced in both cases was similar and resulted from chemical degradation of a histidine residue.

Sequence analysis of these peptides confirmed that the acetylation product was similar in 94–99 and the His-Phe dipeptide and that it resulted from modification of histidine (Table II). The phenylthiohydantoin derivative of the modified histidine residue eluted near PTH-Gly (Figure 4B).

Fast atom bombardment mass spectrometry of the isolated peptides gave M + 1 ions 74 mass units greater than those expected for these peptides containing underivatized histidine (Figure 5A,B). These results again demonstrated that the histidine product was the same in both cases.

Further characterization of the chromophore-containing product was accomplished with ^1H NMR (Figure 6). The

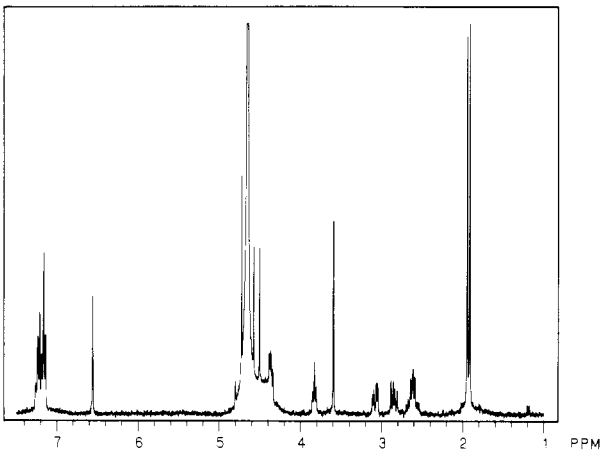

FIGURE 6: ^1H NMR of chromophore-containing synthetic dipeptide. The isolated tryptic peptide of acetylated synthetic Arg-His-Phe which contained chromophore was used at a concentration of approximately 5 mM. The spectrum represents data accumulated from 1000 scans. The methyl protons of incorporated acetate are observed at 1.9–2.0 ppm.

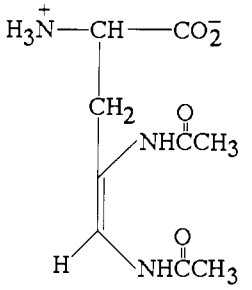

FIGURE 7: Proposed structure of the histidine degradation product.

peptides contained two acetyl groups per histidine residue. The spectrum of the YP?KPE peptide (not shown) was more complex since it contained an N^{ϵ}-acetyllysine and some acetate peaks were split into two populations presumably due to an equilibrium of *cis*/*trans*-proline. Conspicuously absent from these spectra were two signals from the imidazole ring of histidine. Instead, a single uncoupled proton was observed at about 6.6 ppm.

This series of experiments indicated that His-96 of prothrombin fragment 1 became modified during acetylation. The product, which was a chromophore at 250 nm, resulted from the diacetylation of histidine and the subsequent ring-opening reaction. The proposed structure of the histidine degradation product is shown (Figure 7). This structure appeared to be a unique solution to the properties observed.

Reactivity of Histidine-Containing Samples toward Acetic Anhydride. The acetylation of native fragment 1, the isolated 94–99 peptide of fragment 1, and free histidine revealed significant differences in the reactivity of histidine in these substrates (Figure 8). His-96 of native fragment 1 was severalfold more subject to degradation than either the isolated 94–99 peptide or free histidine. In fact, comparison of samples with similar amino acid sequences in the region of interest, native fragment 1 and the 94–99 peptide, demonstrated that the structure supplied by the native peptide made His-96 at least an order of magnitude more reactive toward acetic anhydride. While the differences between native fragment 1 and free histidine were less pronounced, local folding of peptide sequence might make this comparison less significant.

The 94–99 peptide containing acetylated lysine but normal histidine (Table I, peptide C) was examined by ^1H NMR. The two imidazole protons were clearly separated at 7.1 and 8.5 ppm. The signal at 8.5 ppm corresponded to the C_2 proton

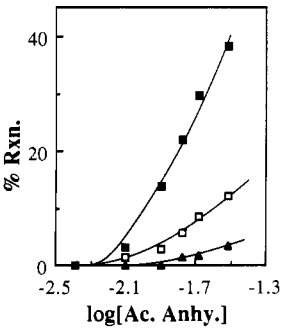

FIGURE 8: Reactivity of histidine toward acetic anhydride as a function of histidine environment. The fraction of histidine degradation resulting from acetylation was measured for native fragment 1 (■), free histidine (□), and the isolated 94–99 peptide of fragment 1 (△). The yield of histidine degradation, % Rxn., was calculated from the observed increase in light absorbance at 250 nm divided by the increase predicted from an extinction coefficient of 5000 M^{-1} cm^{-1} for the degraded histidine. Samples were treated with hydroxylamine (0.2 M, 25 °C for 2 h) prior to absorbance measurements. All samples were derivatized in acetate–borate buffer, and all titrations were performed at peptide concentrations of 43 μM.

since its intensity decreased upon heating in D_2O (Markley, 1975). The mass spectrum of this peptide gave an M + 1 ion at 811 mass units, which is the anticipated mass.

Discussion

These studies indicated that acetylation of prothrombin fragment 1 resulted in the specific irreversible degradation of His-96. The product from this derivatization is shown in Figure 7. The product, like *N*-acetylimidazole, was a chromophore with an absorbance maximum at approximately 250 nm. However, unlike *N*-acetylimidazole, the product was stable to hydroxylamine treatment. A similar histidine degradation has been reported during acylation with diethyl pyrocarbonate (Avaeva & Krasnova, 1975; Miles, 1977). The intermediate monosubstituted histidine derivative shows an increase in absorbance at 250 nm (Ovadi et al., 1967). Therefore, this alteration appears to be generally applicable for severe acylation reactions. Like the dicarbethoxyhistidyl derivative, the disubstituted acetylation product (Figure 7) had a molar extinction coeffecient greater than the monosubstituted histidine. Amino acid and sequence elution profiles allow easy identification of this acetylated derivative.

More importantly, the specificity and reactivity demonstrated by His-96 revealed unusual chemical properties. In this reaction, His-96 became quantitatively converted to chromophore while His-105 was not derivatized to a detectable extent. The primary structure of fragment 1 suggests that these two histidines are spatially close. Consequently, high reactivity was localized to a short segment of the peptide which might result from peptide folding. Cursory examination of the X-ray crystallographic structure (Tulinsky & Park, 1986) did not reveal the basis for such a striking difference in reactivity. However, a recent report by Harlos et al. (1987) indicated that the carbohydrate attached to Asn-101 was entirely ordered while that attached to Asn-77 appeared to be disordered. Since fragment 1 contains other unusual structures (Gla), it is possible that folding of the amino-terminal 35 amino acids accounts for differential reactivity. However, others have suggested that kringle structures represent an independent structural and functional domain and that other regions of the protein should have little impact on the kringle (Castellino et al., 1981; Trexler & Patthy, 1983). The latter is supported by observations with isolated kringles.

Considerable investigation of other kringle structures has shown that the analogous histidine residue also displays un-

usual chemical properties. For example, ^{1}H NMR studies have shown that the imidazole protons associated with this homologous histidine are reversed in their chemical shift positions for kringles 1 and 4 of plasminogen (Hochswender et al., 1983; Llinas et al., 1983; Trexler et al., 1983). These chemical shift abnormalities were initially attributed to ring current effects of other aromatic amino acids. Recently, the dependence of these resonances on pH has suggested that this histidine is in the neighborhood of carboxyl groups (Motta et al., 1986). Photooxidation studies by Lerch and Rickli (1980) suggested that these histidines were important to the lysine binding function of kringles (Wiman & Collen, 1978; Thorsen et al., 1981). Therefore, while the functional involvement of these histidines is not generally agreed upon, it is clear that histidines in the analogous position of various kringles display unusual properties.

One mechanism for generating increased histidine reactivity and increased nucleophilicity would be participation in a charge-relay system. For example, investigations of angiotensin II have shown that the tyrosine hydroxyl and imidazole side chain of this peptide acetylate and deacetylate at markedly higher rates than the corresponding free amino acids (Moore, 1985). The increased nucleophilicities observed were attributed to formation of a tyrosine hydroxyl–histidine–carboxylate charge-relay system. Comparison of the primary sequence of angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) with the isolated 94–99 peptide (Tyr-Pro-His-Lys-Pro-Glu) of fragment 1 reveals similar functional group placement. However, the isolated 94–99 peptide no longer displays the increased nucleophilicity exhibited by intact fragment 1 (Figure 8) and has normal proton resonance shifts. It is tempting to speculate that a charge-relay system existed in fragment 1 but that the carboxyl group was removed during isolation of the 94–99 peptide. In any event, it seems clear that histidines in homologous positions in several kringles show very unusual properties.

It is clear that unusual chemical reactivity extends to other amino acids in the region of His-96. For example, Lys-97 of prothrombin fragment 1 has been shown to be unusually reactive. Chemical modifications designed to specifically derivatize Gla residues have been shown to derivatize Lys-97 as well (Pollock et al., 1988). The procedure involves the addition of morpholine and formaldehyde to a solution of protein at pH 5.0 (Wright et al., 1984). It was assumed that the amino groups of the protein would be protonated at this pH and that imine formation would be negligible. However, isolation of radiolabeled amino acids revealed that Lys-97 had been derivatized. Additionally, mild reductive methylation of fragment 1 using the method of Jentoft and Dearborn (1979) also demonstrated the high reactivity of Lys-97 (Pollock et al., 1988).

Arg-32 of kringle 4 of plasminogen, the amino acid analogous to Lys-97 of prothrombin, was shown to be the most chemically reactive arginine of this kringle (Trexler et al., 1982). Treatments with 1,2-cyclohexanedione demonstrated that Arg-32 was the most reactive but that it did not serve as the critical positive charge in the lysine binding site. Thus, any basic amino acid adjacent to the unusually reactive histidine residue also appears to be activated.

The unusual reactivities exhibited by these amino acids of kringle structures are clearly important to more than the lysine binding site. First, these amino acids appear to be unusually reactive in kringle structures that bind lysine as well as those that do not. This hypothesis is also supported by the recent novel acetylation of Asn-101 of prothrombin fragment 1 (Welsch & Nelsestuen, 1988b). In this case, incorporation of an acetyl group on the β-amide nitrogen of this carbohydrate-linked amino acid abolished the metal ion induced conformational change that caused substantial quenching of fragment 1 fluorescence. These studies demonstrated that the unusual chemical reactivity exhibited by an amino acid in this region of a kringle was correlated to a function other than lysine binding. Therefore, it appears reasonable to suggest that this highly reactive and localized region of kringle structure may serve several important functions.

Overall, it is clear that unusual reactivity extends from at least His-96 to Asn-101. Other functional groups in this region include Glu-99, Tyr-95, and Ser-102. Further studies will be needed to more thoroughly map the extent and to find the function of this highly unusual peptide region that appears common to several kringle structures.

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Abnormal Response to Calmodulin in Vitro of Dystrophic Chicken Muscle Membrane Ca^{2+}-ATPase Activity†

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ABSTRACT: A skeletal muscle membrane fraction enriched in sarcoplasmic reticulum (SR) contained Ca^{2+}-ATPase activity which was stimulated in vitro in normal chickens (line 412) by 6 nM purified bovine calmodulin (33% increase over control, $P < 0.001$). In contrast, striated muscle from chickens (line 413) affected with an inherited form of muscular dystrophy, but otherwise genetically similar to line 412, contained SR-enriched Ca^{2+}-ATPase activity which was resistant to stimulation in vitro by calmodulin. Basal levels of Ca^{2+}-ATPase activity (no added calmodulin) were comparable in muscles of unaffected and affected animals, and the Ca^{2+} optima of the enzymes in normal and dystrophic muscle were identical. Purified SR vesicles, obtained by calcium phosphate loading and sucrose density gradient centrifugation, showed the same resistance of dystrophic Ca^{2+}-ATPase to exogenous calmodulin as the SR-enriched muscle membrane fraction. Dystrophic muscle had increased Ca^{2+} content compared to that of normal animals ($P < 0.04$) and has been previously shown to contain increased levels of immuno- and bioactive calmodulin and of calmodulin mRNA. The calmodulin resistance of the Ca^{2+}-ATPase in dystrophic muscle reflects a defect in regulation of cell Ca^{2+} metabolism associated with elevated cellular Ca^{2+} and calmodulin concentrations.

Biochemical changes described in genetically dystrophic chicken skeletal muscle include abnormal accumulation of calcium (Hudecki et al., 1983, 1984), increased total lipid content in dystrophic sarcoplasmic reticulum (SR)[1] (Scales et al., 1977; Tovar et al., 1983), decreased SR Mg^{2+}-dependent, Ca^{2+}-stimulatable ATPase (Ca^{2+}-ATPase) activity (Hanna & Baskin, 1978; Verjovski-Almeida & Inesi, 1979; Hanna et al., 1981), and increased Ca^{2+}-activated neutral protease activity (Sugita et al., 1982). The molecular mechanisms for these alterations are unknown.

We have recently reported that, compared to normal chickens (line 412), dystrophic chickens (line 413) have affected striated muscle that contains increased amounts of immunoassayable and bioactive calmodulin (Hudecki et al., 1986), a cytoplasmic Ca^{2+} binding protein which governs the

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[1] Abbreviations: SR, sarcoplasmic reticulum; Ca^{2+}-ATPase, Ca^{2+}-stimulatable, Mg^{2+}-dependent ATPase; Mg^{2+}-ATPase, Mg^{2+}-dependent ATPase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CAPP, 2-chloro-10-(aminopropyl)phenothiazine; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; ANOVA, analysis of variance; T-tubule, transverse tubule.

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