Carbohydrate-Linked Asparagine-101 of Prothrombin Contains a Metal Ion Protected Acetylation Site. Acetylation of This Site Causes Loss of Metal Ion Induced Protein Fluorescence Change†

Dean J. Welsch and Gary L. Nelsestuen*

Department of Biochemistry, College of Biological Sciences, The University of Minnesota, St. Paul, Minnesota 55108

Received January 11, 1988; Revised Manuscript Received March 3, 1988

ABSTRACT: Prothrombin fragment 1 (prothrombin residues 1–156) contains two acetylation sites that are protected from derivatization by calcium. The first site was protected by only calcium [Welsch, D. J., & Nelsestuen, G. L. (1988) *Biochemistry* (second of three papers in this issue)] while the second site was protected by magnesium as well. To identify this second acetylation site, fragment 1 was first acetylated with unlabeled reagent in the presence of magnesium. Metal ions were removed, and the protein was acetylated with radiolabeled reagent. The incorporated radiolabel was stable over long periods of time and at acidic or basic pH as long as elevated temperatures were avoided. The radiolabel was removed by treatment of the protein at pH 10 and 50 °C or with 0.2 M hydroxylamine at 50 °C for at least 30 min. Proteolytic degradation of the protein showed that the radioactivity appeared in a tryptic peptide corresponding to residues 94–111 of prothrombin. The Lys-97 in this peptide was acetylated but did not contain radiolabel. Amino acid sequence analysis revealed that the radiolabel was associated with an unextracted sequence product. Aglycofragment 1, produced by treatment of fragment 1 with HF, was radiolabeled by this procedure; peptide 94–111 was isolated and was further digested with protease. The major radiolabeled product contained Asn^{101}-Ser^{102} along with the expected chitobiose attached to Asn-101. NMR analysis revealed the presence of three acetate groups which would correspond to two from the chitobiose plus the incorporated acetate residue. Mass spectral analysis showed the correct mass for this glycopeptide plus a single added acetyl group. Amide ^1H NMR analysis showed only three amide protons rather than the anticipated four. The acetate was not located on either of the α-amino groups of the amino acids. On the basis of these several observations, it is postulated that the site of acetylation is the β-amide nitrogen of Asn-101. The other asparagine-linked carbohydrate site of fragment 1, Asn-77, was not acetylated. Consequently, these studies showed an unusual chemical reactivity in prothrombin fragment 1. They further show that metal ion binding to prothrombin fragment 1 and subsequent protein fluorescence quenching involve sites on the kringle region of the protein.

γ-Carboxyglutamic acid (Gla)[1] residues are required for calcium binding to the vitamin K dependent proteins of the plasma and for subsequent membrane binding by these proteins. Calcium binding to malonic acid derivatives such as that provided by the Gla side chain is of relatively low affinity (Martin, 1984), and it was found that native protein structure was essential to form the tighter calcium binding sites of prothrombin (Nelsestuen et al., 1975). The presence of 10–12 Gla residues in the amino-terminal 40 residues of these proteins would provide intense negative charge. However, efforts to obtain small peptides which bind calcium and subsequently associate with membranes have been unsuccessful. Nelsestuen et al. (1980) reported that minor modifications of the fragment 1 molecule resulted in loss of membrane binding ability. Others (Marsh et al., 1981) have isolated the amino-terminal 39 residues of prothrombin but were unable to definitively show membrane binding. A preliminary report suggests that a peptide corresponding to the amino-terminal 45 residues may associate with membranes (Pollock et al., 1987). Nevertheless, the most consistent observation is that complex protein structure is essential to proper calcium binding and that very few alterations in the native structure are tolerated by the metal ion and membrane binding sites of the protein.

In accompanying papers, we show that acetylation sites in the fragment 1 molecule are critical to proper metal ion binding (Welsch et al., 1988). One acetylation site, which is calcium specific, corresponds to the amino group of N-terminal alanine (Welsch & Nelsestuen, 1988). This paper reports investigations of the second acetylation site. This site is protected from chemical derivatization by either calcium or magnesium and is involved in the fluorescence quenching event associated with metal ion binding to fragment 1 (Welsch et al., 1988). By protein degradation, sequence analysis, and other studies, we found that this acetylation site was associated with carbohydrate-linked Asn-101. A number of properties have led us to propose that the acetylation site corresponded to the nitrogen of the β-carboxamide of asparagine. This unexpected finding also indicated that the kringle portion of fragment 1 is important to metal ion binding and/or protein

†Supported in part by Grant HL-15728 (to G.L.N.) from the National Institutes of Health. The NMR and fast atom bombardment instruments were maintained in part by the Agricultural Experiment Station, The University of Minnesota. The plasma desorption mass spectrometer was purchased with Grant RR 02682 from the NIH and is supported by Grant GM32982.

[1] Abbreviations: fragment 1, amino-terminal 156 amino acids of bovine prothrombin; TFA, trifluoroacetic acid; Gla, γ-carboxyglutamic acid; GlcNAc, *N*-acetylglucosamine; HPLC, high-performance liquid chromatography; FAB/MS, fast atom bombardment mass spectra; PTH, phenylthiohydantoin.

0006-2960/88/0427-4946$01.50/0

fluorescence quenching so that diverse regions of the molecule are essential to proper function.

Materials and Methods

Materials. Isolation of prothrombin fragment 1 has been described in detail in an accompanying paper (Welsch et al., 1988). Aglycofragment 1 was produced by treatment of fragment 1 with anhydrous HF followed by purification as described (Pletcher et al., 1980). All reagents were reagent or HPLC grade. Trypsin (type XIII), protease from *Streptomyces griseus* (type XIV), and V8 protease from *Staphylococcus aureus* (type XVII) were purchased from Sigma. The [^{14}C]acetic anhydride was from Amersham and had a specific activity of 25 mCi/mmol. If not specified here, procedures and methods are given in Welsch et al. (1988).

Acetylation Conditions. Fragment 1 (7.5 mg/mL) was acetylated at ambient temperature in 1.0 M sodium acetate–0.25 M borate buffer (pH 8.5). This was accomplished by addition of a 5-fold molar excess of acetic anhydride (reagent to amino group ratio). After 0.5 h, [^{14}C]acetic anhydride (25 mCi) was added to derivatize remaining groups. Alternatively, the fragment 1 solution was first acetylated in the presence of 25 mM magnesium ion (acetic anhydride to amino group ratio of 50:1). After dialysis to remove metal ions, the peptide was treated with [^{14}C]acetic anhydride (25 mCi). In both cases, excess cold acetic anhydride (1000 equiv) was added at the end of the reaction to ensure derivatization of all groups. Samples were finally dialyzed into 25 mM ammonium bicarbonate buffer (pH 7.5) and lyophilized.

Barium Citrate Adsorption. Peptides (1–5 μM) tested for adsorption to barium citrate were dissolved in 50 mM sodium citrate (pH 7.5). The solution was made 0.2 M in barium chloride, and after thorough mixing at 4 °C, the supernatant and pellet were separated by centrifugation. After the precipitate was washed with 0.1 M sodium chloride (three washes), the pellet was treated with 3 mL of 1.0 M sodium sulfate. The counts in the supernatant and resuspended pellet were then measured.

Reduction and Iodoacetamidination of Disulfides. Reduction of disulfide bonds was performed in 25 mM ammonium bicarbonate buffer (pH 7.8) by addition of dithiothreitol. The final concentration of reducing agent was always at least 10-fold greater than the protein concentration. After at least 1 h at 37 °C, iodoacetamide (iodoacetamide to dithiothreitol ratio of 5:1) was added, and the mixture was incubated for at least 1 h at 37 °C. Excess reagents were removed by dialysis or gel filtration chromatography.

HPLC Separations. Peptide mixtures 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 a 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 shorter peptides (program 1) consisted of aqueous phase (0–5 min), a linear gradient of increasing organic phase to 5% (5–20 min), increasing linear gradient to 15% organic phase (20–30 min), and 30% organic phase at 40 min. The program used for longer peptides (program 2) 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 30% organic phase at 49 min.

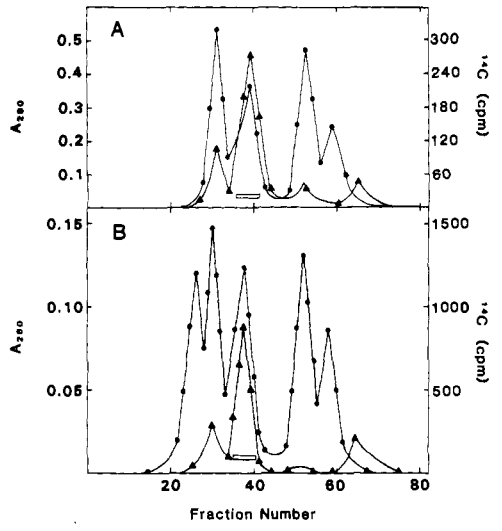

FIGURE 1: Magnesium-protected group is the last group derivatized. The tryptic digestion products of acetylated fragment 1 radiolabeled at the least reactive site were fractionated on Sephadex G-50 (2.0 × 96.0 cm) (A). The tryptic peptides which resulted from acetylated fragment 1 radiolabeled at the magnesium-protected site were fractionated by using the same column (B). The absorbance at 280 nm (●) and radioactivity (▲) of selected fractions are given.

Plasma Desorption Mass Spectrometry. The masses of enzymatically derived peptides from fragment 1 were determined by using plasma desorption time of flight mass spectrometry. The spectra were recorded on a Bioion 10 (Bio-Ion Nordic AB, Uppsala, Sweden) ^{252}Cf plasma desorption mass spectrometer. The spectra were collected for 3×10^6 stop counts with automatic computer background subtraction. The spectra presented have been calibrated on the H^+ and Na^+ peak centroids.

GlcNAc Analysis. Samples used for GlcNAc analysis were hydrolyzed in 3 M HCl at 100 °C under nitrogen for 3 h in sealed tubes. The products formed were analyzed by the procedure outlined by Davidson (1966). Quantitation was achieved by comparison to standards.

^{1}H NMR Spectra. Details of acquisition of the nonexchangeable ^{1}H NMR spectra have been given in Welsch and Nelsestuen (1988). The amide ^{1}H NMR was performed as outlined by Hore (1983) using a 1331 solvent suppression sequence. The difference spectrum of 10% and 99.96% D_2O was obtained.

Fast Atom Bombardment Mass Spectrometry. Fast atom bombardment mass spectra (FAB/MS) were obtained by using 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.

Results

Isolation of Peptides Radiolabeled at the Magnesium-Protected Site. Peptides were selectively radiolabeled in two ways (described under Materials and Methods). Fragment 1 that had been radiolabeled with acetic anhydride at the least reactive site was digested with trypsin and chromatographed on Sephadex G-50 (Figure 1A). Fragment 1 containing radiolabel at the magnesium-protected site was also digested with trypsin and chromatographed (Figure 1B). The same peptide had been labeled in both cases (Figure 1A,B).

Table I: Amino Acid Sequence Analysis[a]

	Sample 1: Pooled Radiolabeled Fractions (2 nmol)[b] sequence turn							
	1	2	3	4	5	6	7	filter[c]
^{14}C (cpm)	≤10	≤10	≤10	≤10	≤10	≤10	≤10	2500
AA 92–98	S(431)[d]	R(–)	Y(575)	P(721)	H(–)	AcK[e](685)	P(719)	
AA 94–100	Y(572)	P(714)	H(–)	AcK(611)	P(718)	E(498)	I(574)	
AA 76–82	G(531)	N(–)	V(454)	S(223)	V(419)	T(281)	R(–)	

	Sample 2: $Asn(GlcNAc)_2$-Ser Dipeptide (500 pmol) sequence turn			
	1	2	3	4
^{14}C cpm	≤25	≤25	≤25	≤25
AA 101–102	–	S(177)	S(21)	–

	Sample 3: Peptide Corresponding to Amino Acids 100–111 (2 nmol) sequence turn						
	1	2	3	4	5	6	7
AA 100–106	I(1100)	–	S(1655)	T(1284)	T(1485)	H(1159)	P(1147)

[a] Background values for other amino acids (AA) were ≤5% of these values. A dashed line indicates no detected amino acid. Carryover is not shown but amounted to about 10% per turn except for proline where it was about 25%. [b] The quantity of peptide subjected to sequence analysis was determined from radioactivity (4000 cpm total) used and was based on the specific radioactivity of the initial fragment 1 (2000 cpm/nmol). [c] A total of 50 sequence turns were run prior to determination of radioactivity remaining on the filter. Radioactivity is uncorrected for counting efficiency. [d] Numbers in parentheses following a letter represent the number of picomoles of that amino acid released. [e] The PTH derivative of N^{ϵ}-amino-acetyllysine is abbreviated AcK and was quantitated by using the extinction coefficient for PTH-A.

The radiolabel could be removed from fragment 1 by treatments that partially restored metal ion dependent protein fluorescence quenching. Barium citrate precipitate was formed in a solution containing a preparation of fragment 1 that had lost all metal ion induced fluorescence quenching and which contained radiolabel at the magnesium-protected acetylation site (see Materials and Methods). All detectable radiolabel (>95%) bound to the barium citrate precipitate and was removed from the supernatant. A second portion of this protein was incubated for 30 min at 50 °C in 0.2 M hydroxylamine (pH 8.0). This treatment restored 60% of the metal ion dependent fluorescence quenching of fragment 1 (Welsch et al., 1988). Addition of barium citrate showed that 79% of the radiolabel was now found in the supernatant. After this hydroxylamine treatment, the protein was dialyzed and subjected to a second acetylation. As before, acetylation caused complete loss of metal ion induced protein fluorescence quenching (data not shown). These results showed that removal of the acetate by hydroxylamine served to expose the same acetylation site; secondary or irreversible chemical modifications did not appear to be involved in the recovery of fluorescence quenching.

Several experiments showed that the radiolabel was not found at the amino terminal or at lysines-3, -11, -44, or -57. The amino terminal and Lys-3 are in the amino-terminal decapeptide that eluted at fractions 48–56 from the Sephadex G-50 column shown in Figure 1 (Welsch & Nelsestuen, 1988). The lysines at positions 11, 44, and 57 of fully acetylated fragment 1 are in tryptic peptides that are disulfide linked to the Gla-containing peptide and would therefore adsorb to barium citrate and elute as the largest peptide on the G-50 column (fractions 26–34). The radiolabeled tryptic peptide did not adsorb to barium citrate (>90% of the radiolabel remained in the supernatant). The only remaining amino group was Lys-97. We proposed that this was the acetylation site in a preliminary report (Welsch & Nelsestuen, 1987). The elution position of this labeled peptide (Figure 1B) did not change after disulfide reduction and alkylation (data not shown).

Sequence analysis (Table I) of the pooled radiolabeled fractions from Figure 1B showed the presence of three peptides. Two sequences were from overlapping peptides and corresponded to residues 94–111 and 92–111. Incomplete trypsin digestion at Arg-93 was a common observation in all of the isolations carried out. Approximately equal yield of these peptides suggested the possibility that cleavage at Arg-91 versus Arg-93 was random and that cleavage at Arg-93 only occurred when it preceded cleavage at Arg-91. The third peptide in this mixture corresponded to the second carbohydrate-containing site (amino acid residues 76–82). A high yield of acetyllysine was obtained at the appropriate turns of the sequencer (Table I). However, no radioactivity was released at any of the turns. In fact, after 50 turns, a high yield of radioactivity was retained with unextracted material on the starting filter. Consequently, the radiolabel was associated with a site that was not solubilized during sequence analysis.

Aglycofragment 1 was also radiolabeled at the magnesium-protected site. As expected for peptide 94–111, the radiolabeled peptide now eluted as a smaller peptide (Figure 2A). Treatment with dithiothreitol and iodoacetamide removed contaminating peptides (Figure 2B), and digestion with protease formed smaller radiolabeled species (Figure 2C). The most abundant of the two components was further purified by HPLC and eluted as a single major radiolabeled component (Figure 3).

Characterization of the Radiolabeled Peptide. Amino acid composition analysis showed that the purified radiolabeled peptide contained residues 101–102 of fragment 1 (Asx, Ser) and 2 mol of GlcNAc (Table II). Sequence analysis gave a low yield of serine in turn two (Table I) as expected for a carboxy-terminal amino acid. This expected result indicated an underivatized peptide backbone. In addition, no radioactivity was released with the serine (Table II).

NMR analysis of the dipeptide showed the peaks anticipated for the $Asn(GlcNAc)_2$-Ser dipeptide. Comparison to standards indicated that the anomeric proton of Asn-linked GlcNAc is seen at 4.98 ppm (Vliegenthart et al., 1983), the anomeric proton of the second GlcNAc at 4.48 ppm (Vliegenthart et al., 1983), the β-hydrogens of Asn at 2.7–3.0 ppm, the α-protons of Asn and Ser at about 4.3 ppm, and other carbohydrate protons in the envelope from 3.45 to 3.85 ppm (Vliegenthart et al., 1983). The large singlet at 3.6 ppm was a contaminant from the HPLC solvents used.

An important observation was the signals of three different acetyl groups (1.882, 1.943, and 1.967 ppm). These were

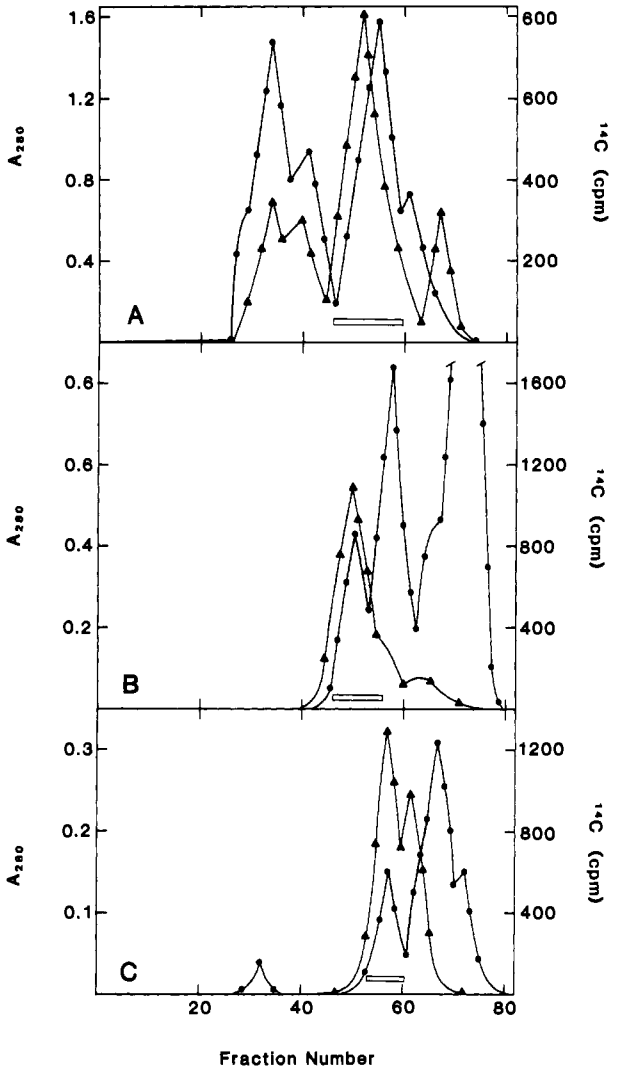

FIGURE 2: Isolation of the radiolabeled magnesium-protected group. The tryptic digestion products from acetylated aglycofragment 1 radiolabeled at the magnesium-protected group were fractionated on Sephadex G-50 (A). The fractions from part A indicated by the bar were pooled, lyophilized, and, after reduction and iodoacetamidination, rechromatographed on the same column (B). The fractions indicated in part B were pooled, lyophilized, and digested with protease prior to rechromatography (C). The absorbance at 280 nm (●) and radioactivity (▲) are given.

present in equimolar ratios and in the correct amounts relative to the other protons in the peptide. Two acetyl groups are expected for the two GlcNAc residues, and one should arise from the radiolabeled acetate.

For comparison purposes, the other carbohydrate-linked peptide (residues 76–82) of acetylated aglycofragment 1 was isolated and studied. Fractions 46–58 of the elution profile in Figure 2A were pooled, concentrated, and subjected to HPLC separation. A well-separated peak (peak half-width equal to 0.5 min) eluting from the HPLC column (same column used in Figure 3) at 20.5 min (program 2) showed the appropriate amino acid composition (Table II). NMR analysis of this peptide showed only two acetyl groups as expected for the two GlcNAc residues (Figure 4B at 1.873 and 1.925 ppm). Comparison of these spectra indicated that the acetyl methyl peak at 1.967 ppm in the Asn(GlcNAc)$_2$-Ser dipeptide (Figure 4A) corresponded to the radiolabeled acetate.

The acetylated Asn(GlcNAc)$_2$-Ser dipeptide was also analyzed for amide protons. The ^{1}H NMR spectrum (Figure 5) showed broad peaks without detectable coupling. However, only two peaks were observed. While quantitation was necessarily approximate, the signals gave relative intensities of about 2:1. The dipeptide should contain four amide protons from the two GlcNAc residues, the Asn–Ser bond, and the Asn side chain. The two equivalent amide protons shown in Figure 5 may arise from the GlcNAc residues. In any event, the major observation was consistent with one fewer amide proton than expected.

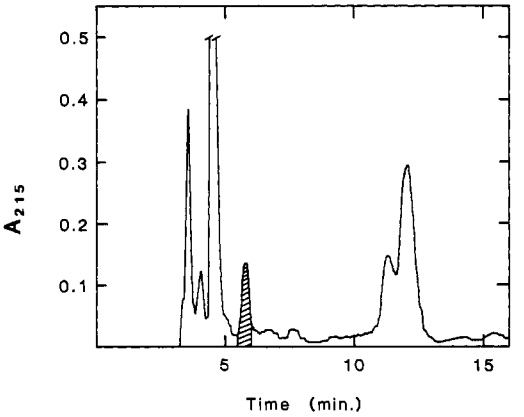

FIGURE 3: Isolation of the radiolabeled dipeptide by HPLC. The major radiolabeled peptide isolated from column C of Figure 2 was further purified by reversed-phase HPLC using program 1. The hatched area of the elution profile indicates the radioactive material pooled. This peak contained 40% of the radioactivity eluting from the column.

Table II: Amino Acid Composition[a]

assignment	peptide 101–102	peptide 76–82
Gly	–[b]	13.00
Asp	2.01	12.81
Val	–	25.23
Ser	1.85	10.78
Thr	–	12.51
Arg	–	12.07
GlcNAc	3.94	+[c]

[a] The values in the table represent the number of nanomoles present for each assignment made. [b] A dashed line indicates that this amino acid was present at background levels. The background values were always ≤10% of those given. [c] A (+) indicates the presence of this component.

The Asn(GlcNAc)$_2$-Ser dipeptide was further analyzed by mass spectrometry (Figure 6). The result showed a M + 1 ion at 668 mass units as well as peaks at 690 and 712 mass units. The latter correspond to the molecular ion plus one and two sodium ions, respectively. The molecular ion corresponds to the mass of a Asn(GlcNAc)$_2$-Ser dipeptide containing exactly one extra acetyl group. This indicated that the peptide did not contain additional, unidentified structures.

Several other experiments were conducted to help define the nature of the acetylation site. First of all, the Asn-(GlcNAc)$_2$-Ser dipeptide was subjected to mild acetylation in aqueous buffer at pH 8. Mass spectra of the product showed an M + 1 ion at 710 mass units and very large ions at 732 and 754 mass units corresponding to M + Na^+ and M + $2Na^+$. This behavior showed addition of exactly one more acetyl group (data not shown) to the amino terminal of the dipeptide. This site was therefore free for acetylation and sequence (Table I).

The experiments shown above indicated that the site of acetylation consisted of an amide nitrogen. The resulting product is an imide. For comparison, the reactivity of a standard imide was investigated. Diacetamide (50 mM) was exposed to 0.2 M hydroxylamine (pH 8.0) at room temperature. Reaction was detected by subsequent addition of 80 mM ferric chloride with measurement of absorbance intensity due

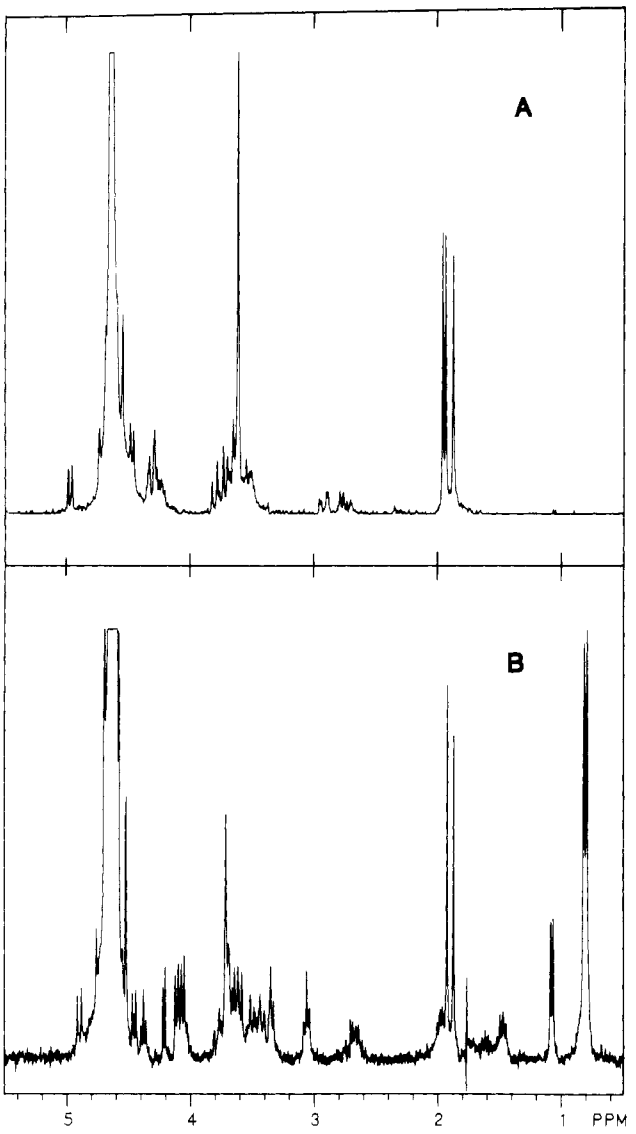

FIGURE 4: ^{1}H NMR spectra of carbohydrate-linked peptides from acetylated fragment 1. The dipeptide isolated by HPLC (Figure 3) was used at a concentration of approximately 0.5 mM. The spectrum represents data accumulated from 1500 scans (A). Part B shows the spectrum obtained from the tryptic peptide corresponding to amino acids 76–82 of acetylated aglycofragment 1. The peptide concentration used in part B was 2 mM, and the spectrum represents data from 1000 scans.

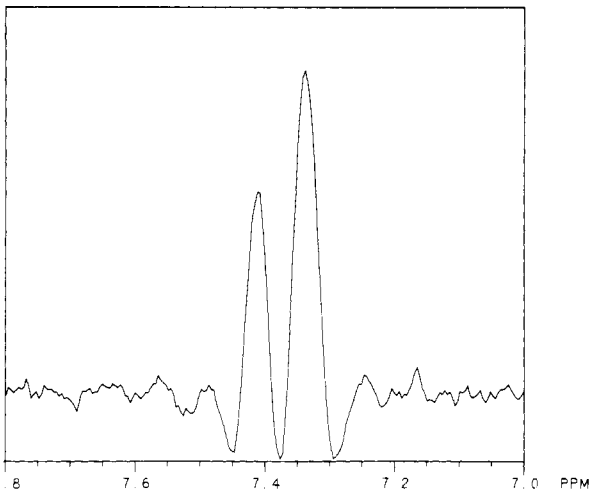

FIGURE 5: Amide ^{1}H NMR spectra of isolated dipeptide. The dipeptide isolated by HPLC (Figure 3) was used at a concentration of approximately 0.5 mM. The spectrum represents data accumulated from 3000 scans. The solvent used was 10% D_2O.

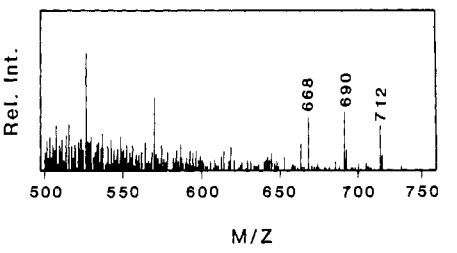

FIGURE 6: Fast atom bombardment mass spectrum of the Asn-$(GlcNAc)_2$-Ser dipeptide.

to the ferric hydroxamate complex at 510 nm. Absorbance intensity of the complete reaction was estimated from a hydroxylamine–diacetamide mixture that was heated at 50 °C for 1 h. At room temperature, the initial reaction rate between hydroxylamine and diacetamide proceeded at about 1% reaction of diacetamide per minute. In contrast, similar reactions with *O*-acetylserine were completed within minutes at room temperature. These results showed that the imide, diacetamide, was much less reactive than an ester but much more reactive than an amide. This general description agrees qualitatively with the stability of the radiolabeled acetate in fragment 1. However, the radioactive acetyl group attached to Asn$(GlcNAc)_2$-Ser was essentially stable to hydroxylamine at room temperature, and only was removed at elevated temperatures. The effect of steric factors and the contribution of the *N*-glycoside bond to stability of an imide are not known.

Acetylation at an amide is unusual and might occur if the native peptide was chemically modified. To test this latter possibility, the peptide corresponding to residues 94–111 of aglycofragment 1 was isolated from magnesium-protected, acetylated protein. The isolation procedure consisted of trypsin digestion and gel filtration on the G-50 column shown in Figure 2. Column fractions 46–58 were pooled and further fractionated by chromatography on HPLC eluted with gradient program 2. A well-separated peptide (peak width 0.5 min) eluting at 27.8 min in this program corresponded in composition to residues 94–111. This material was analyzed by plasma desorption mass spectrometry (Figure 7). The M + 1 ion was observed at 2439.5 mass units which compared favorably with an anticipated mass of 2439.4 mass units. (Calculated molecular weights are based on average isotope abundances.) An additional important ion corresponded to loss of 203 mass units. This arises from loss of one GlcNAc residue, a common fragmentation site. Additional peptides consisting of residues 100–111 were isolated and gave an M + 1 ion at 1688.5 mass units compared to the anticipated mass of 1687.9 mass units (data not shown). These results showed that the unusual reactivity of Asn-101 with acetic anhydride did not arise from an unidentified chemical modification of the native peptide.

A different digestion of acetylated peptide 94–111 was carried out. Radiolabeled peptide from the column shown in Figure 1B was separated from other peptides by chromatography on the HPLC system used in Figure 3 (program 2). The purified single peptide was digested with V8 protease [peptide to enzyme ratio of 100:1 (w/w)] which cleaved at Glu-99. Sequence analysis of this mixture (Table I) showed a high yield of serine at the third turn. This indicated, once again, that the peptide backbone was not the site of acetylation. These combined results indicated that the radiolabeled acetate was associated with the side chain of Asn-101.

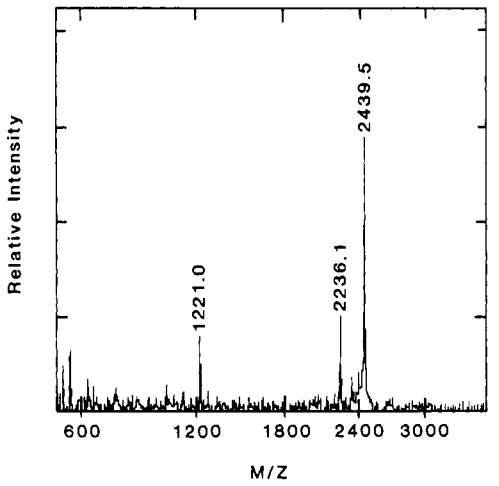

FIGURE 7: Plasma desorption mass spectrometry. Plasma desorption mass spectrum from m/z 1 to m/z 3600 of the tryptic peptide of aglycofragment 1 which contains the magnesium-protected acetylation site. The sample was prepared by nitrocellulose adsorption.

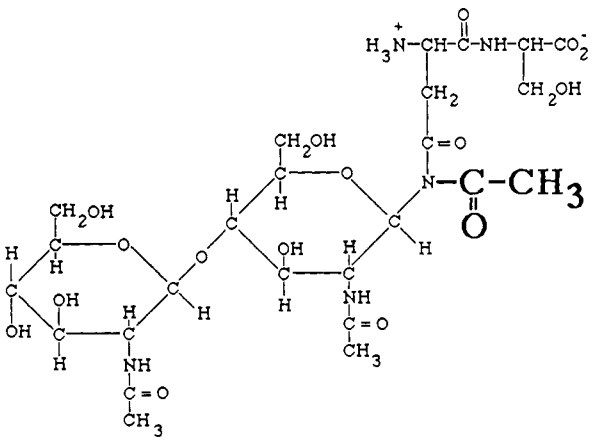

FIGURE 8: Proposed structure. Structure of the isolated dipeptide with added acetyl group on the calcium- and magnesium-protected site.

DISCUSSION

On the basis of the results shown here, we propose that the acetylation site in fragment 1 which is protected by both calcium and magnesium corresponds to the nitrogen of the β-carbamide of Asn-101. This acetylated structure is shown in Figure 8. The chemical stability of the incorporated acetyl group appeared to eliminate ester linkages. In addition, Ser-102 was released as free serine in sequence analysis. Placement of the acetate on the amide of Asn-101 rather than on those of the GlcNAc residues was based on several properties. The amide ^{1}H NMR spectrum indicated two similar amide protons which might correspond to the two GlcNAc residues. In addition, more than half (79%) of the incorporated acetate was released by hydroxylamine treatment. Acetylation at either of the carbohydrate amides would produce a symmetrical imide, and, barring side reactions, a maximum of half of the ^{14}C should be released when all of the imide is cleaved to the hydroxamate plus amide. Acetylation at Asn-101 produces an asymmetric imide. Cleavage at the acetate side of this imide releases acetylhydroxamate while cleavage on the asparagine side of the imide releases the N-acetylated *N*-glycoside. Thus, all of the ^{14}C could be removed from the peptide. Finally, the NMR spectrum showed three distinct peaks, two which correlated precisely with an unacetylated glycopeptide. All of these properties provide strong, although indirect, support for the acetylation site shown.

To our knowledge, an acetylated derivative of this type has not been reported in any other protein and was quite unexpected. While it is difficult to predict the exact effect of the glycoside linkage on the reactivity of this amide nitrogen toward acetic anhydride, it is clear that acetylation was not common to a similar carbohydrate-linked amide group in the same protein. Asn-101 must therefore display unusual chemical reactivity, possibly due to its placement in the native protein structure. While less reactive than amino groups, this site required only 10 times as much acetic anhydride as did lysine residues.

Most importantly, these experiments revealed that metal ions protected a site on the kringle region of prothrombin fragment 1 from acetylation. This would suggest that metal ion binding somehow influences this region of the protein. The mechanism by which acetylation at Asn-101 abolished fluorescence quenching appeared to be related to quenching of the apoprotein. That is, the acetylated apoprotein displayed fluorescence intensity characteristic of the native protein containing metal ions (Welsch et al., 1988). Thus, it is possible that the change in tryptophan environments induced by acetylation is similar to that induced by metal ions. In addition, an ionization event occurring at about pH 10 appeared responsible for a similar fluorescence quenching event (Welsch et al., 1988). This ionization may represent a phenolate anion with lower than average pK. However, since Asn-101 clearly has unusual reactivity, this ionization may be associated with the Asn-101 side chain itself.

Another major conclusion drawn from these studies was that the kringle of the protein was critical to the metal ion induced protein fluorescence quenching event. Therefore, residues that undergo fluorescence quenching may be tryptophans of the kringle (Trp-90 and Trp-126). Previously, Trp-42, which is closer to the amino-terminal Gla region of prothrombin, has seemed the probable candidate for this role. However, these studies make it clear that metal ions may influence the quantum yield of any or all of the tryptophan residues. It is possible that the Gla region of prothrombin is folded in such a manner that allows interaction of Trp-42 with the Asn-101 region of prothrombin. In this context, Asn-101 involvement may be indirect so that conformations produced by the acetylated protein do not allow proper folding of critical regions of the protein. In any event, it is difficult to avoid proposing involvement of the kringle region of fragment 1 in metal ion binding–fluorescence quenching events. Further studies will be needed to clarify this role and to show the relationship between events as Asn-101 and the membrane binding event.

ACKNOWLEDGMENTS

We thank Thomas Krick for assistance in determination of the NMR spectra and the fast atom bombardment mass spectrometry, Dr. Robert Wohlheuter of the Microchemical Facility, The University of Minnesota, for performing the amino acid and sequence analyses, Dr. Paul Lampe for assistance with HPLC analysis, and Dr. Ian Jardine and Dale Scanlon (Mayo Clinic, Rochester, MN) for obtaining the plasma desorption mass spectra.

REFERENCES

Davidson, E. A. (1966) *Methods Enzymol. 8*, 52–60.
Hore, P. J. (1983) *J. Magn. Reson. 55*, 283–300.
Marsh, H. C., Sarasua, M. M., Madar, D. A., Hiskey, R. G., & Koehler, K. A. (1981) *J. Biol. Chem. 256*, 7863–7870.

Martin, R. B. (1984) *Met. Ions Biol. Syst. 17*, 1–49.
Nelsestuen, G. L., Broderius, M., Zytkovicz, T. H., & Howard, J. B. (1975) *Biochem. Biophys. Res. Commun. 65*, 233–240.
Pletcher, C. H., Resnick, R. M., Wei, G. J., Bloomfield, V. A., & Nelsestuen, G. L. (1980) *J. Biol. Chem. 255*, 7433–7438.
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. (1987) *Proceedings of the Seventeenth Steenbock Symposium* (Suttie, J. W., Ed.) June 21–25, 1987, Madison, WI (in press).
Vliegenthart, J. F. G., Dorland, L., & van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–374.
Welsch, D. J., & Nelsestuen, G. L. (1987) *Proceedings of the Seventeenth Steenbock Symposium* (Suttie, J. W., Ed.) June 21–25, 1987, Madison, WI (in press).
Welsch, D. J., & Nelsestuen, G. L. (1988) *Biochemistry* (second of three papers in this issue).
Welsch, D. J., Pletcher, C. H., & Nelsestuen, G. L. (1988) *Biochemistry* (first of three papers in this issue).

Direct Photoaffinity Labeling of Ribonucleotide Reductase from *Escherichia coli* Using dTTP: Characterization of the Photoproducts†

Borys Kierdaszuk‡ and Staffan Eriksson*
Medical Nobel Institute, Department of Biochemistry I, Karolinska Institute, S-104 01 Stockholm, Sweden
Received December 14, 1987; Revised Manuscript Received February 25, 1988

ABSTRACT: Subunit B1 of *Escherichia coli* ribonucleotide reductase contains one type of allosteric binding site that controls the substrate specificity of the enzyme. This site binds the allosteric effector dTTP as well as other nucleoside triphosphates. Cross-linking of dTTP to protein B1 by direct photoaffinity labeling, as well as the isolation and sequence determination of the labeled tryptic peptide, has recently been reported [Eriksson, S., Sjöberg, B.-M., Jörnwall, H., & Carlquist, M. (1986) *J. Biol. Chem. 261*, 1878–1882]. In this study, we have further purified the dTTP-labeled peptide and characterized it using UV spectroscopy. Two types of dTTP-cross-linked peptide were found: one having an absorbance maximum at 261 nm typical for a dTTP spectrum, i.e., containing an intact 5,6 double bond, and one minor form with low absorbance at 261 nm. In both cases, the same amino acid composition was found, corresponding to the peptide Ser^{291}-X-Ser-Gln-Gly-Gly-Val-Arg^{299} in the B1 sequence with X being Cys-292 cross-linked to dTTP. Isotope labeling experiments revealed that one proton in the 5-methyl group of thymine was lost during photoincorporation. Therefore, the cross-linking occurs via the 5-methyl group to Cys-292 in a majority of incorporated dTTPs, but a second, possibly 5,6-saturated form of incorporated nucleotide was also detected. The reasons for the high stereospecificity of the reaction and the possible structure of the allosteric site of protein B1 are discussed.

Photochemistry has provided a successful approach to study protein–nucleotide interactions, since proteins and unmodified nucleotides or nucleic acids cross-link covalently when irradiated with ultraviolet light. This form of direct photoaffinity labeling has been used to investigate the properties of nucleic acid binding proteins such as aminoacyl-tRNA synthetase (Schoemaker & Schimmel, 1974; Schoemaker et al., 1975; Yue & Schimmel, 1977; Schimmel, 1977), DNA and RNA polymerases (Markovitz, 1972; Strniste & Smith, 1974; Hillel & Wu, 1978), RNase A (Sperling & Havron, 1976; Havron & Sperling, 1977), ssDNA binding proteins (Paradiso et al., 1979; Merrill et al., 1984), and recA proteins (Banks & Sedgwick, 1986) as well as many other systems [see Shetlar (1980)].

This technique has also been used in recent years to study several nucleotide binding proteins, e.g., myosin (Maruta & Korn, 1981), ribonucleotide reductase (Eriksson et al., 1982, 1986; Caras & Martin, 1982; Caras et al., 1983; Eriksson, 1983), DNA polymerase (Biswas & Kornberg, 1984), deoxycytidylate deaminase (Maley & Maley, 1982), and terminal deoxynucleotidyltransferase (Modak & Gillerman-Cox, 1982), using both purine and pyrimidine nucleotides as photoaffinity ligands.

Many different amino acids including serine, isoleucine, threonine (Havron & Sperling, 1977), tyrosine (Maly et al., 1980), cysteine, lysine, and arginine (Smith & Meun, 1968; Schott & Shetlar, 1974; Shetlar et al., 1975; Varghese, 1973, 1976; Paradiso et al., 1979) have been shown to be covalently cross-linked to nucleotides in direct photoaffinity labeling reactions. Generally, only those amino acids that are in close proximity to the nucleotide in the native protein structure are supposed to be involved in cross-linking (Shetlar, 1980). However, only in very few instances has the chemical nature of the products of direct photoaffinity reactions been identified.

In this investigation, we have characterized the structure of the covalent linkage between *Escherichia coli* ribonucleotide reductase and dTTP that was obtained by using direct photoaffinity labeling. This enzyme is responsible for the reduction of all four ribonucleotides to the corresponding deoxyribonucleotides (Thelander & Reichard, 1979) needed for DNA

†This investigation was supported by grants from the Swedish Medical Research Council, the Swedish Cancer Society, The Magn. Bergvall Foundation, and the Medical Faculty of the Karolinska Institute.
‡Supported by a grant from the Medical Faculty of the Karolinska Institute. Permanent address: Department of Biophysics, Institute of Experimental Physics, University of Warsaw, 02-089 Warsaw, Poland.

0006-2960/88/0427-4952$01.50/0