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Supplementary Material Available

A table summarizing the basis for amino acid assignments in peptide Tf (1 page). Ordering information is given on any current masthead page.

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Effects of Temperature and pH on Prothrombin Fragment 1 Conformation As Determined by Nuclear Magnetic Resonance†

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ABSTRACT: The effects of temperature and pH on the solution conformation of native prothrombin fragment 1 were examined with ^1H NMR spectroscopy. A calcium-dependent quenching of the intrinsic protein fluorescence was used to monitor calcium binding to fragment 1 as an indicator of functional protein. The native fragment 1 NMR spectrum contained several features indicative of a folded protein: (a) nonequivalent histidyl C-2 resonances at 7.9 and 8.1 ppm, (b) two resonances of nearly equal intensity at 7.26 and 7.32 ppm, and (c) a resonance at −1.04 ppm. Temperature studies showed that thermal unfolding of fragment 1 (even at 80 °C) was reversible; however, there was an irreversible inactivation step which occurred subsequent to the unfolding. The basis for this inactivation appeared to include disulfide exchange reactions. On the basis of NMR spectra, fragment 1 retained its conformation from pH 7.0 to pH 11.5. From pH 7.0 to pH 5.0, the protein showed a reversible conformational change, and below pH 5, the protein self-associated. The pH dependence of the chemical shift of the tyrosyl resonances indicated a pK_a of approximately 10 for the tyrosyl residues. These data suggest that the tyrosyl residues are accessible to solvent in the native protein.

Prothrombin is one of a group of extracellular calcium-binding proteins which undergo a vitamin K dependent carboxylation of glutamyl residues (Stenflo & Suttie, 1977). The resulting γ-carboxyglutamyl residues have been implicated as ligands for calcium in these proteins. Prothrombin fragment 1[1] (prothrombin residues 1–156) contains all the γ-carboxy-

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[1] Abbreviations used: NMR, nuclear magnetic resonance; TSP, sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4; EDTA, ethylenediaminetetraacetic acid; fragment 1, prothrombin residues 1–156; HPLC, high-performance liquid chromatography.

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glutamyl residues in prothrombin and has been proposed as a suitable model peptide for calcium binding to the vitamin K dependent proteins (Gitel et al., 1973; Nelsestuen, 1976; Nelsestuen et al., 1975, 1976; Prendergast & Mann, 1977; Benson & Hanahan, 1975; Dombrose et al., 1979; Jackson et al., 1979; Nelsestuen & Lim, 1977).

Fragment 1 has at least six metal ion binding sites (Nelsestuen et al., 1981). For most metal ions, these sites can be divided into two classes according to the effect on the protein. One class induces a protein conformational change (Nelsestuen, 1976; Furie et al., 1978; Prendergast & Mann, 1977) which can be monitored by changes in the intrinsic fluorescence of both prothrombin and fragment 1. The second class is involved in protein self-association (Nelsestuen et al., 1981). Calcium and magnesium, the physiologically relevant metals, are exceptions. Calcium induces the conformational change and the protein self-association concomitantly whereas magnesium causes the conformational change with very limited self-association (Nelsestuen et al., 1981).

While the occurrence of a metal ion induced conformational change in fragment 1 is well documented (Nelsestuen, 1976; Furie et al., 1978; Prendergast & Mann, 1977; Bloom & Mann, 1978; Nelsestuen et al., 1981), little is known in detail about the structural rearrangements in the protein. Both fluorescence quenching and CD data (Carlisle et al., 1980) indicate that the environment of at least one of the three tryptophans is altered upon metal ion binding. Additional changes in protein conformation involving other aminoacyl residues or segments of the polypeptide chain are not documented.

Because of the small size (M_r 22 500) and the high solubility of fragment 1, high-resolution NMR may provide a direct means for monitoring changes in protein conformation and for identifying metal ion ligands. Examples of previous studies on this system include the work of Furie et al. (1979), who used natural-abundance ^{13}C NMR to examine the effect of lanthanide ions on a small γ-carboxyglutamic acid containing peptide (prothrombin residues 12–44). Their data indicate that γ-carboxyglutamyl carbon atoms are preferentially perturbed by the lanthanide ions and that a high-affinity metal binding site is formed by γ-carboxyglutamic acid residues 15 and 25. In a preliminary, high-resolution ^1H NMR study, Esnouf et al. (1980) tentatively assigned the two lowest field peaks to the two histidyl C-2 protons and reported that native fragment 1 is irreversibly denatured by heat treatment.

Theoretically, the NMR spectrum can give information about the environment of every aminoacyl residue in a protein (Roberts & Jardetzky, 1970). In practice, identification of ligands and changes in protein conformation are determined by comparison of appropriate spectra. Thus, it is essential that the spectrum of the native protein can be identified. In this paper, we identify resonances in the ^1H NMR spectrum of fragment 1 which are indicative of the native folded structure and monitor the effect of temperature and pH on certain resonances. The temperature studies reveal that thermal unfolding of fragment 1 is reversible at short times; however, there is an irreversible step which can occur subsequent to the unfolding. The pH studies show that fragment 1 can be reversibly titrated from pH 5.5 to pH 11.5, and the data suggest that the tyrosines are accessible to solvent.

Experimental Procedures

Protein Preparation. Prothrombin fragment 1 was prepared as described (Heldebrant & Mann, 1973; Pletcher et al., 1980). Protein concentrations were determined by ultraviolet absorbance using $E^{1\%}_{280nm}$ = 10.1 for fragment 1 (Heldebrant & Mann, 1973). Reduced and carboxyamidomethylated fragment 1 was prepared essentially by the method of Henriksen & Jackson (1975). Metal ion free samples of protein were prepared by dialyzing the protein for at least 12 h against 1000 volumes of 0.5 mM EDTA, pH 7.5. Subsequently, the protein was dialyzed against three 2000-volume changes of distilled, deionized water. In all cases, the water was prepurified on a Barnstead reagent-grade water purification system (Boston, MA) to give type I water (<1 ppm metal ion present). For NMR analysis, the protein solution was lyophilized to dryness 2 times from D_2O solution and finally dissolved in D_2O containing 0.1 M NaCl. D_2O (100 mL) (Sigma) was extracted with 2 mL of 0.02% diphenothiocarbazone (Aldrich) in CCl_4 to remove contaminating divalent cations. The D_2O was extracted with additional 5-mL aliquots of CCl_4 until the CCl_4 was colorless. The NaCl was passed through Chelex 100 (Bio-Rad) to remove divalent cations.

pH Titrations. Adjustment of pH in protein solutions were accomplished by adding 0.075 M NaOD or 0.10 M DCl to the samples. pH values were measured on a Radiometer Model 26 pH meter equipped with a Radiometer GK2412C electrode. The pH values reported are not corrected for deuterium isotope effects.

Calcium Binding. Calcium-dependent quenching of intrinsic protein fluorescence (Nelsestuen, 1976) was used as an indication of calcium binding to the protein and of protein function. Several studies have indicated that this fluorescence change is a sensitive monitor of functional prothrombin fragment 1 (Nelsestuen et al., 1980). Unless indicated otherwise, all protein samples for which spectra are presented were fully functional and showed 50% calcium-dependent protein fluorescence quenching at the end of NMR analysis. In this relationship [$(1 - F/F_0) \times 100 = 50\%$], F is the fluorescence intensity in the presence of Ca^{2+} and F_0 is the fluorescence intensity in the presence of excess EDTA. Excitation was at 280 nm, and emission was at 344 nm.

NMR Spectra. Proton NMR spectra were recorded on a Bruker HR 270 spectrometer operating in the Fourier-transform mode. Spectra were routinely obtained by using a 9-μs pulse ($\sim$90°), a 6000-Hz sweep width, and an acquisition time of 1.36 s. Each spectrum reported here represents data accumulated from at least 500 free induction decays containing 16K data points leading to a resolution of 0.75 Hz/point. Exponential apodization equivalent to the line broadening of 1 Hz was used. The temperature of the probe was calibrated using ethylene glycol or methanol (Van Geet, 1968, 1970). Chemical shifts were measured from an external standard of TSP. The residual HDO peak was not saturated, and a resonance at 3.66 ppm was identified as contamination from Tris-HCl, the buffer in which the protein was purified.

Additional Procedures. Molecular weights were determined by relative light scattering with an argon ion laser (Pletcher et al., 1980). Steric exclusion chromatography was performed on a Varian (Palo Alto, CA) Model 5000 liquid chromatograph with a TSK 2000 SW steric exclusion column. A constant flow rate of 1 mL/min of 50 mM Tris–acetate buffer, pH 7.4, containing 0.1 M sodium acetate was used for elution.

Results

The NMR spectrum at 80 °C for the reduced and carboxyamidomethylated prothrombin fragment 1 was typical of that for a random coil protein (Figure 1A) (Roberts & Jardetzky, 1970; Bundi & Wüthrich, 1979). The sharp resonance at 2.06 ppm was previously assigned to the methyl group of *N*-acetylglucosamine (Esnouf et al., 1980). In agreement with this assignment, the ^1H NMR spectrum of deglycosylated

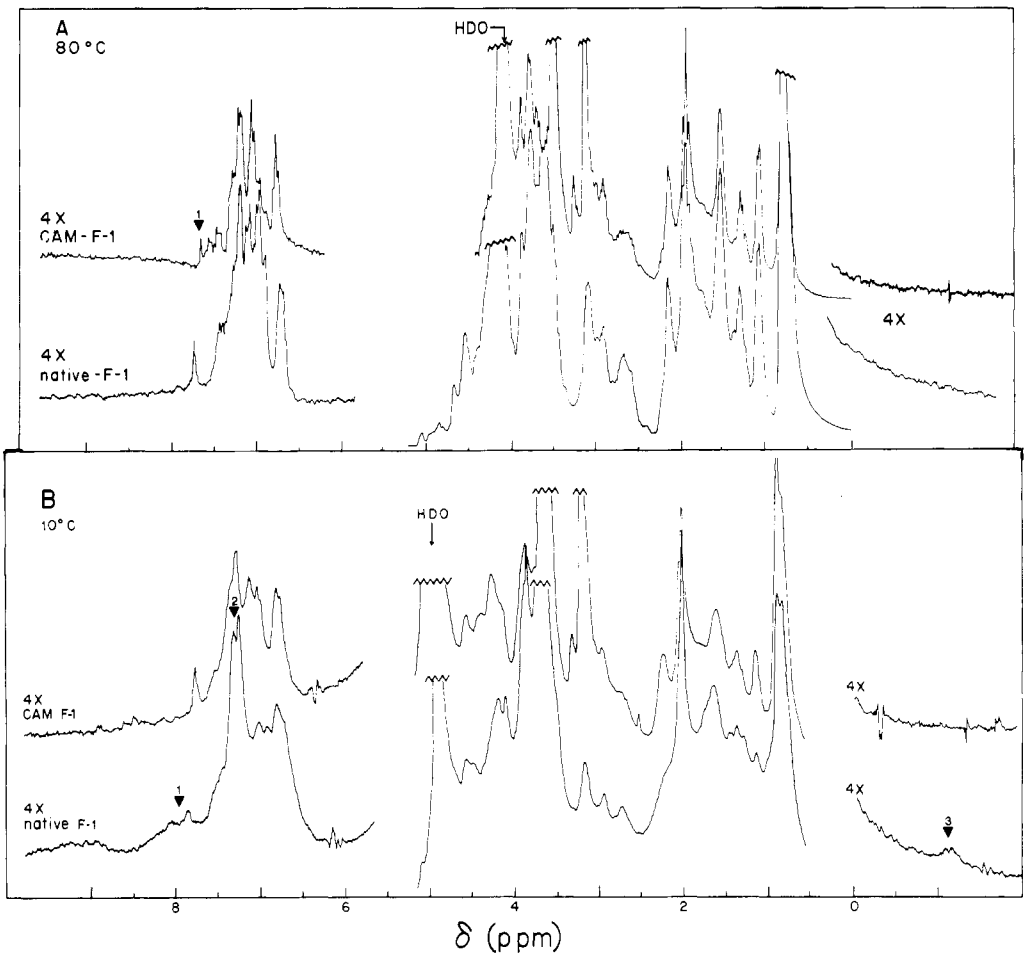

FIGURE 1: ^{1}H NMR spectra (270 MHz) of prothrombin fragment 1 and the reduced and carboxyamidomethylated fragment 1. The spectra were recorded at 80 °C (A) and at 10 °C (B). The solution was 2 mM protein at pH 7.3 with 0.1 M NaCl in D_2O as solvent. CAM F-1 is the reduced and carboxyamidomethylated fragment 1. Arrow 1 indicates the peaks of the histidyl C-2 protons; arrow 2, the aromatic peaks at 7.26 and 7.32 ppm; arrow 3, the peak at −1.04 ppm.

prothrombin fragment 1, prepared as described elsewhere (Pletcher et al., 1980), showed a major reduction of this peak (spectrum not shown). The spectrum of the reduced and carboxyamidomethylated fragment 1 at 10 °C (Figure 1B) was essentially identical with the spectrum at 80 °C except that there was line broadening observed as compared to the high-temperature spectrum.

In contrast to the spectra of the reduced and carboxyamidomethylated protein, the 10 °C spectrum of native fragment 1 (Figure 1B) was unlike the high-temperature spectrum (Figure 1A) and showed characteristics of a folded protein. On the basis of chemical shift (Bundi & Wüthrich, 1979) and exchange with solvent (see below), the peak at 7.67 ppm in the 80 °C spectrum was assigned to the two histidyl C-2 protons. This is consistent with the assignment of Esnouf et al. (1980). In the 10 °C spectrum, the two histidyl C-2 protons were no longer equivalent and were shifted downfield to 7.9 and 8.1 ppm (Esnouf et al., 1980; Figure 1B). Additional significant characteristics in the aromatic region of the folded protein (10 °C spectrum) include two peaks of nearly equal intensity at 7.26 and 7.32 ppm. A new peak at −1.04 ppm was also observed in the spectrum of the folded protein (Esnouf et al., 1980; Figure 1B).

In the 10 °C spectrum of native fragment 1, substantial line broadening relative to the high-temperature spectrum was apparent (compare panels A and B of Figure 1). Attempts were made to determine if this broadening was due to factors other than the molecular size of the protein. It was found that the line widths were essentially independent of protein concentration from 0.3 to 3 mM protein; therefore, protein self-association is not a broadening mechanism in this concentration range. Independent light scattering (Pletcher et al., 1980) and ultracentrifugation studies (Heldebrant & Mann, 1973) support this conclusion. No enhancement in resolution was achieved by the addition of excess EDTA, thereby making the possibility that paramagnetic metal ions were a source of line broadening remote.

As the temperature of the protein sample was increased, there was some enhancement in resolution, indicating increased motion within the protein (Figure 2). In addition, changes in the relative intensities of some aromatic peaks with increasing temperature indicated some unfolding as the temperature was increased to 56 °C. At higher temperatures, denaturation occurred as evidenced by the loss of the features of the tertiary structure which were cited above. Differential scanning calorimetry experiments show that fragment 1 is thermally denatured at 58.5 °C (Ploplis et al., 1981). Following each NMR experiment, an aliquot of protein was removed and assayed at ambient temperature for the calcium-induced protein fluorescence change. In every experiment shown in Figure 2, this assay indicated fully functional protein. The NMR spectra also support reversible thermal unfolding. A comparison of the initial 10 °C spectrum (bottom of Figure 2) and the final 10 °C spectrum (top of Figure 2), collected after cooling the 80 °C sample, showed that the thermal unfolding was essentially reversible.

However, fragment 1 is not stable indefinitely at high temperatures. A time study of irreversible thermal inactivation

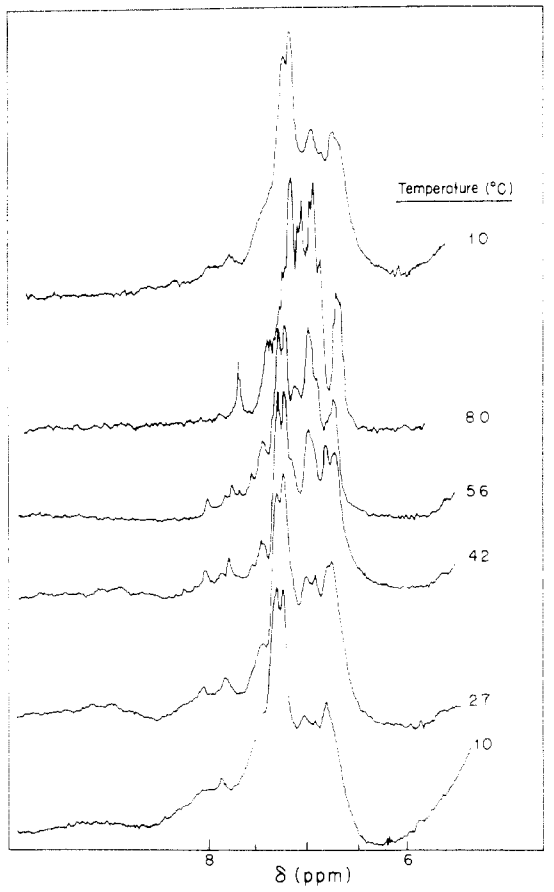

FIGURE 2: Aromatic region of the ^1H NMR spectrum of prothrombin fragment 1 as a function of temperature. The concentration of protein was 4 mM; the pH of the sample was 7.3. For a test of the reversibility of thermal unfolding, the 80 °C sample was cooled to 10 °C; and a final 10 °C spectrum, which is shown at the top of the figure, was recorded.

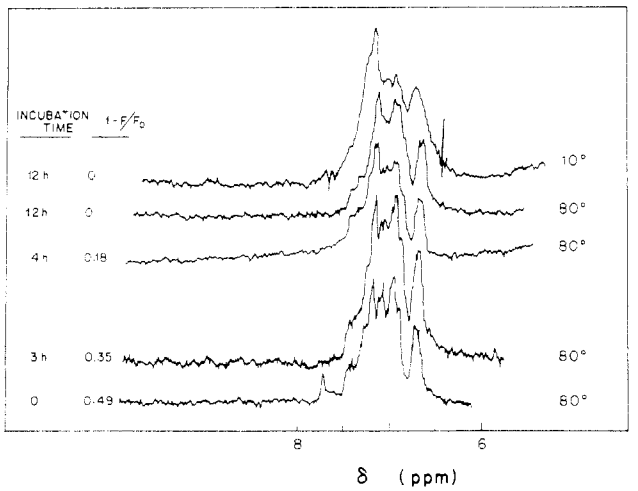

FIGURE 3: Aromatic region of the ^1H NMR spectrum of thermally inactivated prothrombin fragment 1. The concentration of protein was 1 mM, the pH was 7.4, and the incubation temperature was 80 °C. After the spectrum was acquired, aliquots of protein were removed from the NMR sample and subsequently assayed for calcium binding. $1 - F/F_0$ is the calcium-dependent fluorescence quenching which was used as an indicator of calcium binding. F is the fluorescence intensity in the presence of Ca^{2+}, and F_0 is the fluorescence intensity in the presence of excess EDTA. The starting protein showed $1 - F/F_0 = 0.50$. The spectrum at 10 °C at the top of the figure was recorded on the sample incubated at 80 °C for 12 h.

at 80 °C (Figure 3) showed that alterations in the NMR spectrum corresponded to losses in the calcium-dependent intrinsic protein fluorescence quenching. Spectra taken after

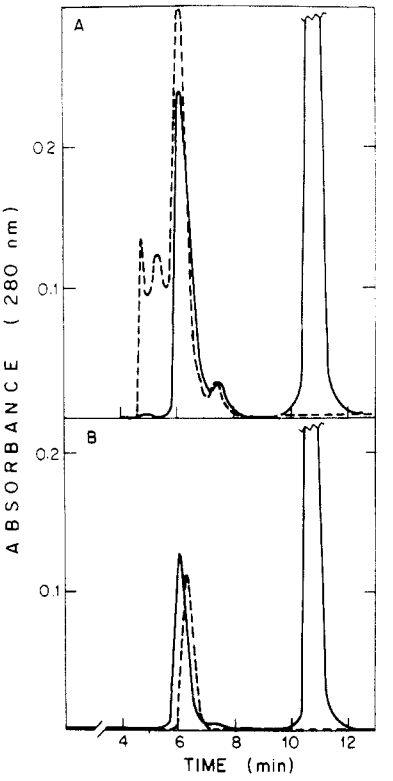

FIGURE 4: HPLC gel permeation chromatography of prothrombin fragment 1. Thermally inactivated protein (A) and native fragment 1 (B) were separated by steric exclusion chromatography. Both nonreduced (- - -) protein and protein reduced (—) with 2% mercaptoethanol at 37 °C for 2 h were separated. The peak at 10.7 min is the mercaptoethanol in the reduced sample.

cooling the sample (10 °C spectrum, Figure 3) indicated that the protein did not refold into the native conformation. Other experiments have shown that inactivation also occurred at lower temperatures but at a slower rate. For example, one protein preparation incubated at 40 °C for 12 h was completely and irreverisbly denatured. Control experiments indicated that the thermal inactivation proceeded in pure D_2O at the same rate as in a Tris-HCl-buffered solution. Moreover, a 10-fold greater volume of D_2O than is routinely required for dissolving the protein was lyophilized to concentrate any nonvolatile residue. The thermal inactivation was independent of the nonvolatile residue.

During thermal inactivation, high molecular weight species of fragment 1 were formed as evidenced by gel filtration chromatography (Figure 4). Reduction of the thermally treated protein with mercaptoethanol returned the protein to the monomeric form. Disulfide exchange may be the primary basis of protein inactivation at elevated temperatures.

Incubation at high temperatures results in the exchange of the histidyl C-2 proton with solvent (Markley, 1975). In the fragment 1 spectrum, the peak at 7.67 ppm has completely disappeared in 3 h when at least 70% of the protein remained functional (Figure 3). On this basis, the peak at 7.67 ppm was assigned to the two histidyl C-2 protons of fragment 1. The loss of intensity of the peak at 7.67 ppm was not proportional to the loss of functional protein, thereby making the possibility that the loss of intensity was due to the chemical shift change caused by denaturation highly unlikely.

Native fragment 1 can be reversibly titrated from pH 3.0 to pH 11.5 (Scott et al., 1979). Between pH 7.3 and 11.5, the NMR data showed no changes in the chemical shift or the relative intensities of the −1.04-ppm peak (not shown), the histidyl C-2 proton peaks, or the 7.26- and 7.32-ppm peaks

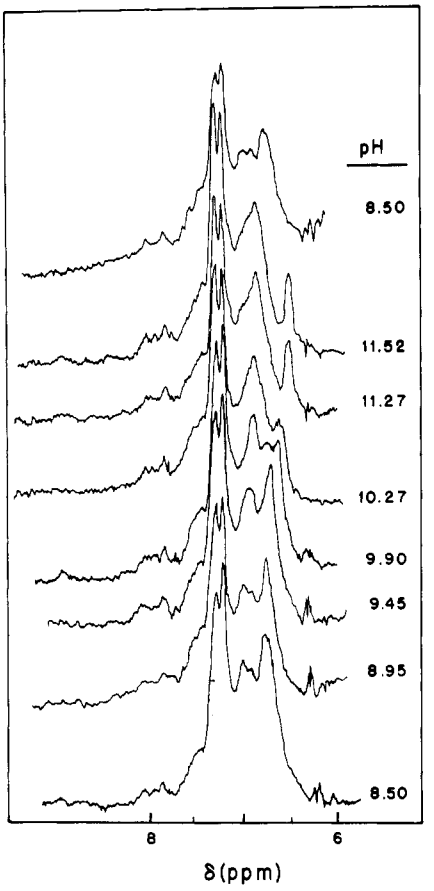

FIGURE 5: Aromatic region of the ^{1}H NMR spectrum of prothrombin fragment 1 at 10 °C. The protein concentration was 1 mM; the pH of the sample was as indicated. The pH of the pH 11.52 sample was adjusted to 8.50 and a final spectrum (top) was taken to show total reversibility of the titration.

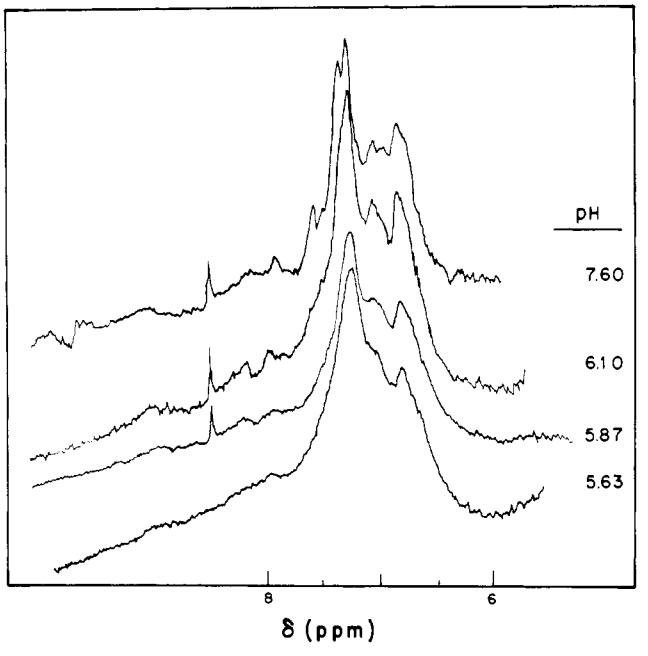

FIGURE 6: Aromatic region of the ^{1}H NMR spectrum of prothrombin fragment 1. The concentration of protein was 1 mM, the pH of the sample was as indicated, and all spectra were recorded at 10 °C. The sharp resonance at 8.45 ppm is due to a contaminant.

(Figure 5). In addition, the circular dichroism spectrum of fragment 1 at pH 11 was indistinguishable from the circular dichroism spectrum at pH 7.5 (not shown). The protein therefore appears to remain folded in essentially the same

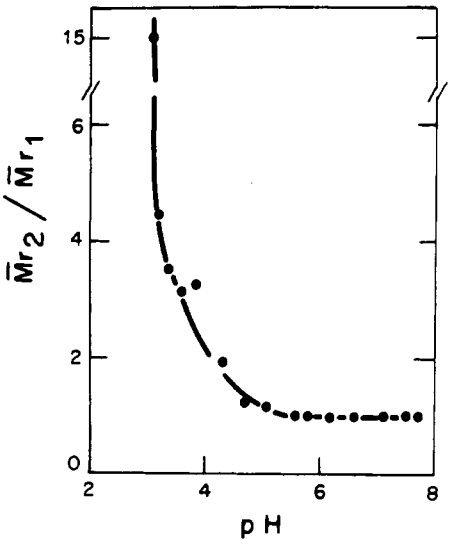

FIGURE 7: Protein molecular weights measured by relative light scattering. $\bar{M}_{r_2}$ is the average molecular weight of the protein at the indicated pH; $\bar{M}_{r_1}$ is the molecular weight of the protein at pH 7.0 which was 23 500. The concentration of protein was 0.14 mM.

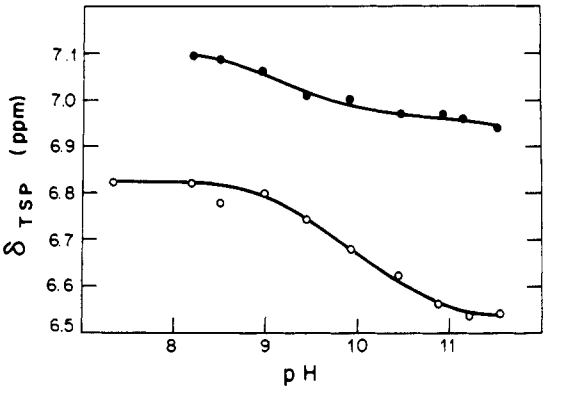

FIGURE 8: pH dependence of the chemical shifts of tyrosine resonances. The chemical shifts for the resonance of the 3,5 protons (○) were determined directly from the spectra in Figure 2. The chemical shifts for the 2,6 protons (●) were identified by using difference spectra (King & Bradbury, 1971). The spectrum at pH 8.5 was subtracted from the other spectra (Figure 5).

conformation from pH 7.3 to pH 11.5. In contrast, at acidic pHs, the intensities of the −1.04-ppm resonance and the histidyl C-2 proton resonances were drastically reduced, and the two resonances at 7.26 and 7.32 ppm were substantially broadened (Figure 6). The possibility that the additional peak broadening at low pH was due to protein self-association was investigated by using relative light scattering to determine protein molecular weight (Figure 7). No self-association was detected above pH 5. Below pH 5, self-association occurred, and at pH 3, the protein aggregation was extensive.

Tyrosine resonances show a characteristic shift during pH titration (Bundi & Wüthrich, 1979). On the basis of the chemical shift, the peak at 6.81 ppm was tentatively assigned to the 3,5 protons of tyrosyl residues (Bundi & Wüthrich, 1979). This peak showed a characteristic upfield shift of 0.28 ppm with a pK_a of 10 during pH titration (Figure 8). A peak which could be tentatively assigned to the 2,6 protons of tyrosyl residues was not clearly resolved in the full spectrum. However, difference spectra were used to identify pH-dependent peaks. A peak which titrated with a pK_a of approximately 10 and which shifted from 7.06 to 6.94 ppm during the titration was clearly distinguished in the difference spectra (King & Bradbury, 1971; Figure 8).

Discussion

The NMR spectrum of native prothrombin fragment 1 at 10 °C contains several features which are indicative of a folded protein with tertiary structure. First, especially evident, is a peak at −1.04 ppm which is absent in the spectrum of the reduced and carboxyamidomethylated protein (Esnouf et al., 1980; Figure 1) and which disappears from the spectrum of the native protein at high temperature. Hence, this peak arises from the conformation of the protein. Similar peaks, observed in the spectrum of lysozyme, have been assigned to methyl groups which experience a ring current shift in the folded protein (McDonald & Phillips, 1967). Second, at high temperature or in the reduced and carboxyamidomethylated protein, the two histidyl C-2 protons are equivalent. In the low-temperature spectrum, the histidyl C-2 resonances are shifted downfield and are no longer equivalent. That the two histidyl residues do not experience the same magnetic environment is evidence for tertiary structure in a protein. Third, the relative intensities of two peaks at 7.26 and 7.32 ppm change with the conformation of the protein. Even though this region of the spectrum represents the superposition of several peaks, it is still quite useful for identifying the spectrum of the folded protein.

These three characteristics of the folded protein can be used to monitor protein structure under various conditions. Substantial changes which result from increased motion within the protein occur in the NMR spectrum as the temperature is raised from 10 to 56 °C; yet, the protein does not denature (Figure 2). The thermal denaturation temperature of fragment 1 is 58.6 °C (Ploplis et al., 1981). The protein can be reversibly denatured at higher temperatures without a substantial deleterious effect on either the solution conformation of the protein as determined by ^{1}H NMR or the calcium-induced protein conformation change. The latter was monitored by changes in protein fluorescence in the presence of calcium. However, fragment 1 will undergo irreversible disulfide exchange reactions if the unfolded protein is maintained at elevated temperatures (Figure 4). This irreversible inactivation is a concern even at moderate temperatures, and continual evaluation of the protein function is necessary for useful application of NMR to prothrombin fragment 1. Esnouf et al. (1980) reported that thermal unfolding of fragment 1 was irreversible. Indeed, their spectrum of fragment 1 at 80 °C shows strong similarities to our spectrum of fragment 1 which had undergone irreversible disulfide exchange (Figure 3).

Examination of the NMR spectrum as a function of pH shows that the protein remains folded between pH 7.5 and 11.5 (Figure 5). In particular, the resonance at −1.04 ppm, the nonequivalent histidyl C-2 resonances at 7.9 and 8.1 ppm, and the two resonances at 7.26 and 7.32 ppm are retained. At pH values below 7.0 (Figure 6), the spectra broaden significantly. A conformational change was reported to occur in human fragment 1 between pH 4.9 and 5.9 (Gabriel et al., 1975). For the bovine protein, the additional line broadening in the NMR spectrum at low pH may be due to a similar conformational change. It is also possible that protein self-association which was not detected by light scattering could contribute to line broadening.

Relative light-scattering studies did show that below pH 5.0 fragment 1 self-associates in a pH-dependent manner (Figure 7). These studies indicate that attempts to use NMR to monitor carboxylate titrations or to measure exchange of amide protons at acidic pH will be hampered by the protein self-association. The light-scattering studies were limited to protein solutions less than 0.2 mM protein. Thus, self-association would be expected to be even more extensive at the 10-fold higher concentration of protein used for NMR studies.

The NMR spectra provide some insight into the structural environment of the tyrosines. Unlike the tyrosine resonances of the bovine pancreatic trypsin inhibitor (Karplus et al., 1973), resonances from individual tyrosine residues in prothrombin fragment 1 are not clearly resolved (Figure 5). Titration between pH 8.5 and 11.5 provides no additional resolution of the peaks. These data suggest that the tyrosine residues of fragment 1 are in similar environments with characteristics analogous to tyrosine in a tetrapeptide (Bundi & Wüthrich, 1979). For example, the chemical shifts of tyrosine resonances in the tetrapeptide were 7.15 and 6.86 ppm for the protonated form and 6.98 and 6.57 ppm for the deprotonated form. These values correlate well with those observed in fragment 1 (Figure 8). The pK_a for tyrosine in a tetrapeptide, 10.3 (Bundi & Wüthrich, 1979), is also quite similar to the pK_a of approximately 10 (Figure 8) observed for tyrosines in fragment 1. These data suggest that all four tyrosines in fragment 1 are relatively accessible to solvent and are not constrained in a polar bond. Since the protein does not unfold as the tyrosines are titrated, these amino acids may be on the surface of the protein.

Acknowledgments

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Nuclear Magnetic Resonance Studies of the Role of Histidine Residues at the Active Site of Rabbit Muscle Creatine Kinase†

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ABSTRACT: Fourier transform proton NMR studies at 250 MHz using 16-bit A/D conversion allowed detection of six imidazole C-2 proton resonances and one imidazole C-4 proton resonance of the 16 histidine residues per subunit of rabbit muscle creatine kinase (M_r 82 000). Titrations measuring their chemical shifts as a function of pH* yielded pK' values of 7.0, 7.1, 5.9, and 5.2 for His-2, -3, -4, and -6, respectively, and permitted the assignment of the C-4 resonance to His-3. The pK' of His-2 was unaffected by creatine but was increased by 0.6–0.7 unit on saturation of the enzyme with the phosphorylated substrate phosphocreatine or MgATP, in quantitative agreement with the results of a pH–rate study of creatine kinase [Cook, P. F., Kenyon, G. L., & Cleland, W. W (1981) *Biochemistry 20*, 1204], indicating that His-2 is the general acid/base catalyst which deprotonates the guanidinium group of creatine as it is phosphorylated by MgATP. The pK' values of His-4 and His-6, while too low to fit the kinetic data as the general acid/base catalyst, also increased in response to the binding of phosphorylated substrates. The pK' value of His-3 was not greatly altered by substrate binding, suggesting that His-3 is not directly involved in catalysis. Titrations of creatine kinase with substrates at constant pH* (6.8) monitoring the chemical shifts of His-2 or His-6 yielded dissociation constants for phosphocreatine and MgADP consistent with those derived from kinetic data, indicating active-site binding. The dissociation constant of MgATP so determined agrees with the results of other binding studies but is an order of magnitude lower than kinetically determined K_D values, suggesting a steady-state random kinetic scheme in the forward direction. The exchange rate of MgADP from its creatine kinase complex (350 s^{-1}) determined by NMR line broadening is 5-fold greater than k_{cat}, indicating a kinetically competent E·ADP·Mg^{2+} complex. Direct evidence for the presence of His-2, His-3, and His-6 at or near the active site was provided by the paramagnetic effects of the substrate analogue β,γ-bidentate Cr^{3+}ATP on the longitudinal and transverse relaxation rates of their imidazole protons. The longitudinal relaxation rates yielded distances of 12 Å from Cr^{3+} to the C-2 protons of His-2 and His-6, consistent with His-2 functioning as the general acid/base catalyst and with His-6 interacting electrostatically with the substrates. His-3 is somewhat farther from Cr^{3+}ATP and is so positioned that its C-4 proton is oriented toward the Cr^{3+}, at a distance of 14 Å. His-4 is too far from the Cr^{3+}ATP (≥18 Å) for a paramagnetic effect to be observed, suggesting that the increases in its pK' values induced by the binding of substrates result from indirect conformational effects.

Creatine kinase is a dimeric enzyme (M_r 82 000) which catalyzes the reversible phosphorylation of creatine by Mg^{2+}ATP. While much information exists on the kinetic mechanism (Morrison & James, 1965; Morrison & Cleland, 1966; Schimerlik & Cleland, 1973; Hammes & Hurst, 1969), the ligands of the divalent cation activator (Reed & Leyh, 1980), and the nature of certain amino acid residues at or near the active site, less is known about the precise roles of these residues in substrate binding and catalysis.

A sulfhydryl group, implicated in the catalytic mechanism as a result of complete enzyme inactivation by iodoacetamide (Watts & Rabin, 1962), was found to be nonessential by the use of the smaller sulfhydryl reagent, methyl methanethiosulfonate (Smith & Kenyon, 1974). At the nucleotide binding site, an arginine residue was detected by chemical modification studies with diacetyl (Borders & Riordan, 1975) and by nuclear Overhauser (NOE)[1] studies of the adenine protons of

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