Analysis and Identification of Aromatic Signals in the Proton Magnetic Resonance Spectrum of the Kringle 4 Fragment from Human Plasminogen†

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Received July 6, 1984

ABSTRACT: The aromatic ^{1}H NMR spectrum of the kringle 4 domain from human plasminogen has been reexamined in order to identify signals stemming from individual residues. Acid–base titration, nuclear Overhauser effect experiments, and two-dimensional correlated spectroscopies have been implemented in order to analyze the spectrum both in the presence and in the absence of ligands. All six histidyl imidazole singlets have been recognized and paired according to their common side-chain origin. A similar identification has been achieved for the three sets of tryptophanyl resonances, and for Trp-I, the correspondence between indole singlet and multiplets is unambiguously established. The single phenylalanyl side chain and all tyrosyl phenol spin systems have been identified. Titration experiments indicate that one or two of the tryptophans are in the vicinity of carboxyl groups. It is shown that the spectrum for one tyrosyl ring, Tyr-V, undetectable at ~300 MHz, becomes visible at 600 MHz, reflecting slow motion on the NMR time scale and a constrained location within the kringle. A simulation of the complete kringle 4 aromatic spectrum is included.

Human plasminogen contains five highly homologous kringles (Sottrup-Jensen et al., 1978) that behave as independent structural and folding domains (Castellino et al., 1981; Trexler & Patthy, 1983). Kringles 1 and 4 can be isolated intact by controlled proteolysis of the plasminogen heavy chain and have been studied by high-resolution ^{1}H NMR[1] spectroscopy (De Marco et al., 1982; Trexler et al., 1983; Hochschwender et al., 1983; Llinás et al., 1983). Motivation for this research is stimulated by a lack of a three-dimensional structure for kringle-containing polypeptides: although a low-resolution X-ray crystallographic study has been published for fragment 1 of prothrombin, the reported model is not sufficiently detailed to reveal conformational features of the kringle (Olsson et al., 1982). Furthermore, NMR spectroscopy can provide valuable insights into the dynamics of kringles in solution and their mode of binding ligands such as lysine and other ω-amino acid analogues, some of which have been evaluated as antifibrinolytic drugs.

In previous papers, common conformational features were established for human plasminogen kringles 1 and 4 (Llinás et al., 1983) and for the latter and the bovine prothrombin fragment 2 (Trexler et al., 1983). The ^{1}H NMR studies centered on the close structural homology among the kringles and on the identification and assignment of resonances on the basis of amino acid substitutions and chemical modifications. Although the two independent studies (Llinás et al., 1983; Trexler et al., 1983) concurred on most aspects of the analysis of the kringle 4 ^{1}H NMR spectrum, there were discrepancies regarding the identification of spin systems linked to His-II (His-C), His-III (His-A), and Trp-II (Trp-B), where Roman numerals are used following Llinás and collaborators and capital letters (in parentheses) as used by Trexler and coinvestigators. Furthermore, the tyrosyl and single phenylalanyl aromatic spectra were only partially revealed. This paper represents a common effort of the European and American groups to resolve the ambiguities and to extend and further substantiate the identification and assignment of aromatic resonances for kringle 4, exploiting 2-D NMR spectroscopy, NOE experiments, and acid–base titration studies.

Materials and Methods

Plasminogen was digested with pancreatic elastase to generate fragments containing the various kringles, and kringle 4 was purified by affinity chromatography on lysine–Sepharose according to the described procedure of Sottrup-Jensen et al. (1978). The ligand 4-(aminomethyl)bicyclo[2.2.2]octane-1-carboxylic acid (AMBOC) was a gift from Dr. R. Hirschman (Merck Sharp & Dohme).

Proton NMR spectra were recorded in the Fourier mode at 300 MHz on a Bruker WM-300 spectrometer, at 470 MHz on an Oxford Enzyme Group spectrometer, and at 600 MHz on the NMR Facility for Biomedical Studies at Carnegie-Mellon University. Kringle 4 solutions were ~0.5 or ~5 mM in COSY experiments, in ^2H_2O, the solvent originating from Merck Sharp & Dohme of Canada, Ltd. Dioxane was used as internal reference standard and assumed to resonate at 3.766 ppm from sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate (De Marco, 1977). Resolution enhancement was achieved by

† This research was supported by the U.S. Public Health Service, NIH Grants HL 29409 and HL 15535. The 600-MHz NMR facility is supported by Grant RR 00292 from the National Institutes of Health.
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[1] Abbreviations: AMBOC, 4-(aminomethyl)bicyclo[2.2.2]octane-1-carboxylic acid; BASA, *p*-benzylaminesulfonic acid; COSY, two-dimensional correlated spectroscopy; NOE, nuclear Overhauser effect; pH*, glass electrode pH reading uncorrected for deuterium isotope effect; ppm, parts per million; SECSY, two-dimensional spin-echo correlated spectroscopy.

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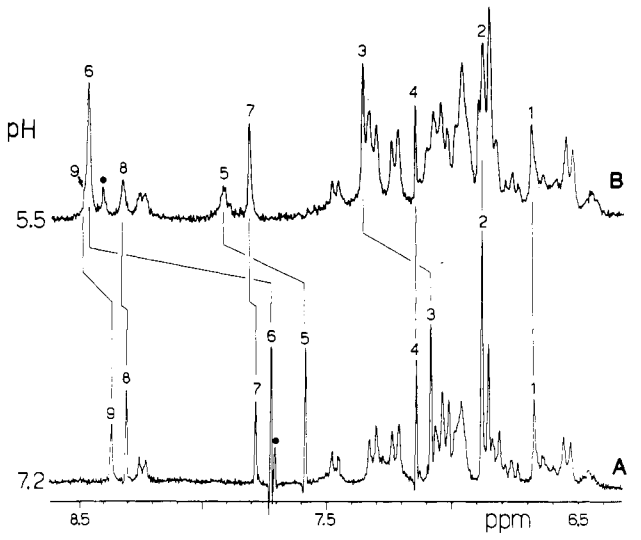

FIGURE 1: ^{1}H NMR spectra of kringle 4: acid–base perturbation of the aromatic resonances. The spectra were recorded at 300 MHz, 27 °C, after exchange of labile hydrogen atoms for deuterium: (A) pH* 7.2; (B) pH* 5.5. Histidyl and tryptophanyl singlets are numbered according to their chemical shift ordering at pH* 7.2 (A). A titratable peak of reduced intensity (●) is attributed to singlet 6 arising from a minor species of kringle 4 containing the extra dipeptide segment -Ser^{87}-Val^{88} at the C-terminus (Hochschwender et al., 1983).

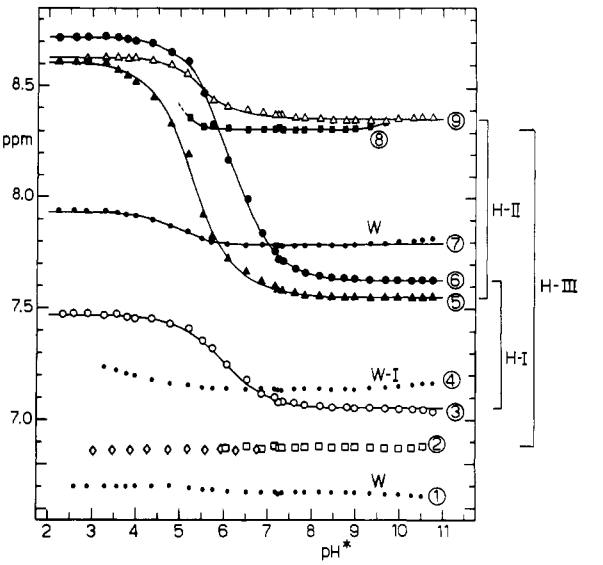

FIGURE 2: Acid–base titration of singlets in the aromatic ^{1}H NMR spectrum of kringle 4. Singlets are numbered as in Figure 1. The experimental data points are based on spectra recorded at 300 MHz: circles, His I; triangles, His-II; squares, His-III; open symbols, H4 resonances; filled symbols, H2 resonances. Dots indicate data points for Trp H2 resonances. Solid line result from nonlinear regression fits to the Henderson–Hasselbach equation. The derived pK_a values are as follows: 6.1 ± 0.1, His-I; 5.4 ± 0.1, His-II; 4.8 ± 0.2, Trp singlet 7. Diamonds denote data points for pH titration of His-III at 600 MHz in the presence of BASA.

Gaussian convolution (Ernst, 1966; Ferrige & Lindon, 1978). The PANIC program, Bruker Aspect 2000 data package, was used for spectral simulation.

Acid–base titration curves were analyzed on the basis of the equation

$$\delta = \frac{\delta_{HA} + 10^{s(pH-pK_a)}\delta_{A^-}}{1 + 10^{s(pH-pK_a)}} \quad (1)$$

where the two parameters s and pK_a were fitted by nonlinear regression. In eq 1, δ, δ_{HA}, and δ_{A^-} are the observed, protonated acid, and conjufate base chemical shifts, respectively, and s is the Hill coefficient (Markley, 1975).

SECSY spectra were acquired with the modified SECSY-45° program (Bruker Aspect 2000 data package). A data acquisition matrix ($\nu_1 \times \nu_2$) of 256 × 2048 points was zero filled to 512 × 4096 before Fourier transformation. Resolution enhancement was via Gaussian convolution in both dimensions. NOE spectra were obtained with a 1-s presaturation pulse.

RESULTS

The amino acid sequence of kringle 4 contains three His and three Trp residues (Sottrup-Jensen et al., 1978) whose carbon-bound aromatic protons resonate in the low-field, 9 ppm > δ > 5.5 ppm, spectral region (Bundi & Wüthrich, 1979). In particular, resonances from His imidazole H2 and H4 and Trp indole H2 atoms appear as sharp singlets, since these protons exhibit only small spin–spin coupling to other nuclei, which is generally not demonstrated in routine one-dimensional spectra. The aromatic region of the kringle 4 ^{1}H NMR spectrum at 300 MHz, 25 °C, pH* 7.2, is shown in Figure 1A. The expected nine singlets from the His and Trp side chains are clearly discerned in the spectrum and have been numbered 1–9 according to their order of appearance following the chemical shift ppm scale (Llinás et al., 1983). Furthermore, while pK_a ~ 6 is observed for an unhindered His imidazolium group, a free Trp indole side chain does not titrate. Figure 1 illustrates the spectral response on going from pH* 7.2 to pH* 5.5: clearly, singlets 1, 2, and 4 do not sense the increase in acidity, and therefore, they might arise from indole or buried imidazole groups. From Figure 1, we also notice that singlets 3, 5, 6, and 9 shift significantly more than 7 and 8 do and that singlets 5, 8, and 9 broaden.

Complete acid–base titration curves based on spectra at 300 MHz are shown for the nine singlets in Figure 2, where each curve is numbered according to the convention used in Figure 1A. It is noted that singlet 1 does not exhibit any significant acid–base response in the range 2.5 < pH* < 11 and is therefore assigned to a Trp residue. We also identify singlet 4 as arising from a Trp side chain (Trp-I): it is insensitive to additions of alkali up to pH* 10.5 although it shows a minor shift when the acidity is increased below pH* ~5.5. For singlet 2, it proved problematic to unambiguously monitor its titration below pH* 6 owing to signal overlap in a crowded spectral region. However, this difficulty could be circumvented by studying the spectrum of the BASA complex of the kringle, which exhibits an overall resonance narrowing (Hochschwender et al., 1983), and also by resorting to spectroscopy at 600 MHz to improve the spectral dispersion. Singlet 2 is relatively insensitive to the presence of BASA, a ligand that interacts with the lysine binding site of kringle 4 and perturbs its proton spectrum (Hochschwender et al., 1983; Llinás et al., 1983). Such experiments indicate a lack of response of singlet 2 in the complex to acidification down to pH* 3 and some protection of this side chain by the bound ligand. This result suggested initially that singlet 2 arises from the third tryptophanyl side chain, but other experiments (described below) showed this not to be the case.

In order to identify conclusively the three pairs of histidine singlets in the spectrum, a COSY experiment was carried out with an additional 50-ms delay. This approach produces cross-peaks between the histidine H2 and H4 resonances by virtue of their small coupling constants (King & Wright, 1982), but with the protein dissolved in ^2H_2O and the indole NH protons exchanged, the tryptophan H2 resonances still behave as singlets. Figure 3 shows selected cross-sections from the data with a sample of kringle 4 in the presence of the ligand

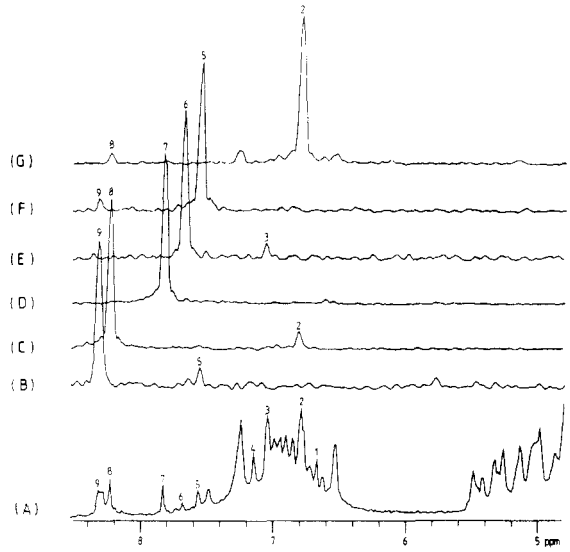

FIGURE 3: Identification of histidyl and tryptophanyl ^1H NMR singlets at 470 MHz. (A) Aromatic region of the spectrum of kringle 4 containing the ligand AMBOC. The positions of the nine singlets are marked in accord with Figure 1. (B–D) Cross-sections taken from the COSY experiment with an additional 50-ms delay (see text). Each section contains a single intense peak that represents the diagonal element of the two-dimensional array. The smaller peaks are the cross-peaks arising from resonances coupled to the large resonance in the same section.

AMBOC; the positions of the singlets in the spectrum in this sample are indicated. Cross-peaks are clearly seen connecting resonances 5 and 9 (His-II), 2 and 8 (His-III), and even 3 and 6 (His-I), despite resonance 6 being considerably diminished in intensity due to deuteration of the His-I H2 position [Markley (1975) and references cited therein]. The magnitude of the pH* dependence of resonance 6 confirms its assignment as the H2 proton of His-I with a pK_a* of 6.1 ± 0.1. Similarly, the size of the pH*-dependent shift of 5 shows it to arise from the H2 proton of His-II. Its H2 origin was confirmed by its diminution in intensity as deuterium exchange of the proton occurred at 70 °C. Under the same conditions, resonance 8 is also seen to be diminished in intensity and is therefore assigned to the H2 proton of His-III. This identification is in accord not only with the chemical shifts of resonances 2 and 8 and those expected for a histidyl residue (Bundi & Wüthrich, 1979) but also with the direction and magnitude of the shift that resonance 8 shows on a lowering of the pH*.

From the above, it follows that singlet 7 must be assigned to a Trp indole, despite the fact that this resonance exhibits a titration curve with pK_a* = 4.8 ± 0.2. This effect might be caused by proximity of the indole (ring) to a titrable side-chain group such as free carboxylic acid from Asp or Glu residues. It is also seen in Figure 3 that singlet 7 is not coupled to any other resonance. The complete aromatic spectrum of kringle 4 at 300 MHz is depicted in detail in Figure 4. The 2-D SECSY experiment (Figure 4B) reveals scalar connectivities for the Trp multiplets. The latter, together with the His singlet pairing established above, are also indicated in the conventional 1-D spectrum (Figure 4A). Of the indole resonances, only singlet 1 has been assigned to a specific residue, namely, Trp^{72} (Trp^{71}), on the basis of chemical modification experiments and comparative spectroscopy with kringle 1 (De Marco et al., 1982; Trexler et al., 1983; Hochschwender et al., 1983; Llinás et al., 1983). It is interesting that each Trp residue exhibits one triplet (indole H5 or H6) resonance at ~5 ± 0.2 ppm, a rather high-field position that most likely results from close interaction among aromatic side chains.

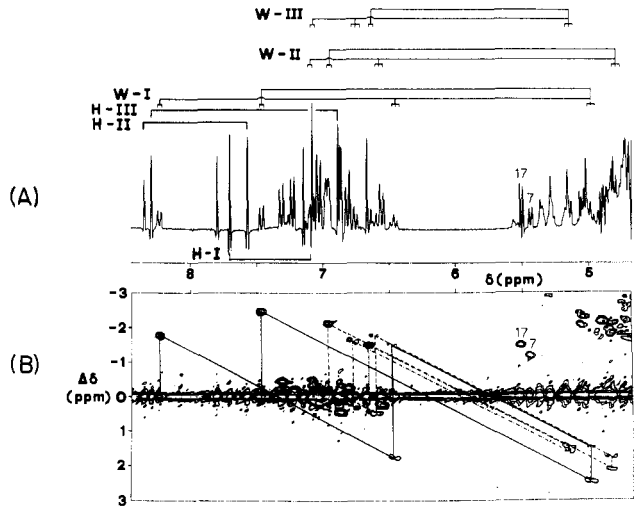

FIGURE 4: ^1H NMR spectra of kringle 4: identified histidyl and tryptophanyl aromatic spin systems at 300 MHz. Spin–spin connectivities, derived as indicated from the 2-D SECSY spectrum (B), are schematically represented in the conventional, 1-D spectrum (A). Indole CH resonances of Trp-I (—), Trp-II (– –), and Trp-III (–·–) are shown connected. The $^1H^2HO$ line was irradiated for 1 s between scans, pH* 7.2, 45 °C.

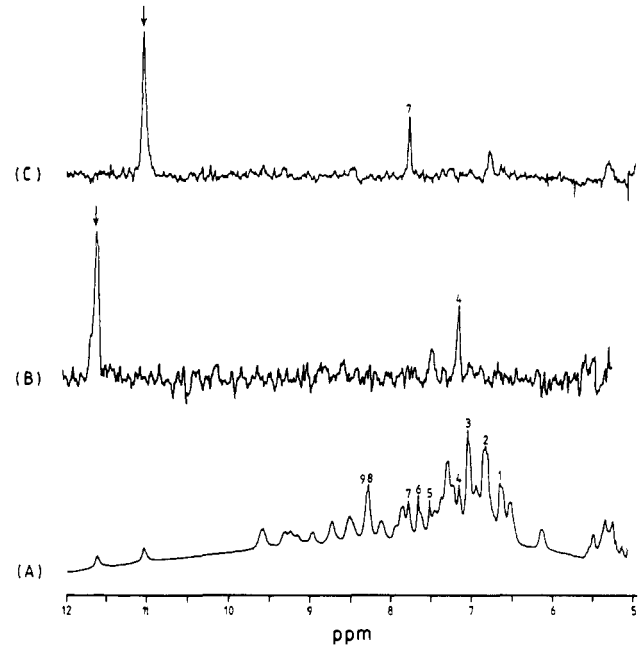

FIGURE 5: Downfield region of the 300-MHz kringle 4 spectrum, pH* 8.3, 27 °C: (A) normal spectrum with the singlets marked; (B) and (C) NOE difference spectra after a 1-s irradiation, the arrowhead indicating the Trp indole NH resonance irradiated. Samples of spectra A and C contained the ligand 6-aminohexanoic acid.

[Two α-CH doublets near 5.5 ppm are also detected in the SECSY spectrum as part of the threonyl residues with γ-CH_3 groups labeled 7 and 17 in a previous paper (Llinás et al., 1983).]

Figure 5B shows a NOE experiment on a NH resonance at 11.6 ppm. It is characteristic of the enhancement expected on irradiation of a tryptophan ring NH, giving the H2 (singlet 4, 7.12 ppm) and H7 (doublet, 7.43 ppm) proton resonances in the difference spectrum (Poulsen et al., 1980). This identifies the complete Trp-I indole ring spectrum. Figure 5C shows a similar experiment with presaturation of the NH resonance at 11.1 ppm, although this sample contained the ligand 6-aminohexanoic acid. It is seen that singlet 7 is present in the difference spectrum, which confirms its indole origin.

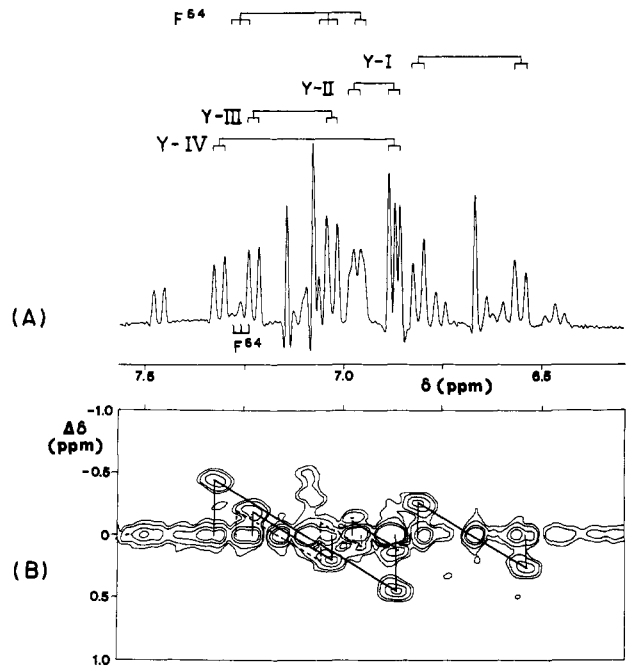

FIGURE 6: ^{1}H NMR spectra of kringle 4: identified phenylalanyl and tyrosyl spin systems at 300 MHz. Spin–spin connectivities are derived as indicated in the 2-D SECSY spectrum (B) and schematically represented in the conventional 1-D spectrum (A). Broken lines connect the phenylalanyl one-proton triplet with (tentative) a two-proton triplet and a two-proton doublet (see Figure 7). Solid lines link doublet pairs of Tyr AA′XX′ phenolic spin systems. This figure is an expansion of Figure 4.

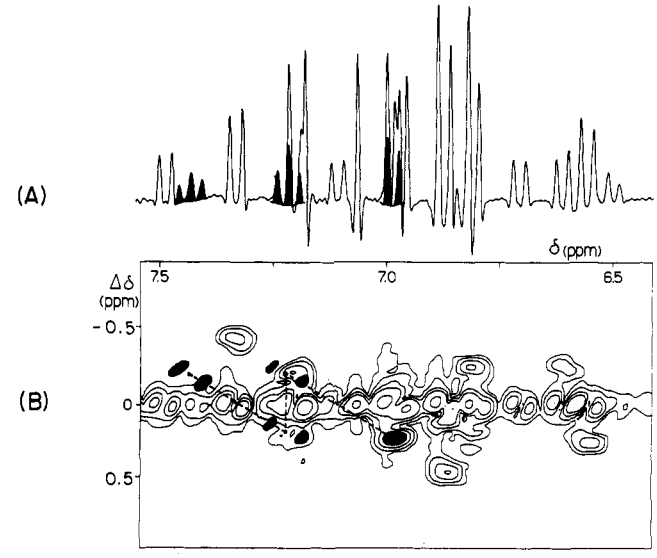

FIGURE 7: Identification of aromatic phenylalanyl resonances: 300-MHz spectra of kringle 4 in the presence of 2-fold excess BASA. (A) The 6.5–7.5 ppm spectral region. Phenyl resonances are shown shaded. (B) SECSY spectrum. Phenyl aromatic spin–spin connectivities link, from low to high fields, the one-proton H4 triplet, the two-proton H3,5 triplet, and the two-proton H2,6 doublet. This spectrum was recorded at 55 °C, pH* 7.2, after exchange of labile hydrogen atoms for solvent deuterium.

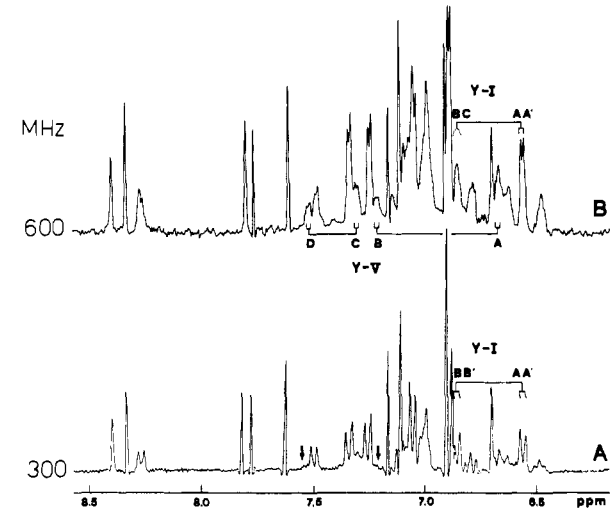

FIGURE 8: ^{1}H NMR spectra of kringle 4: magnetic field effects on the aromatic resonances. The spectra were recorded at 300 (A) and 600 MHz (B) to detect time scale effects on the tyrosyl resonances. Tyr-I doublets are identified based on the basis of data shown in Figure 6. The Tyr-V resonances can be detected at 600 MHz, at which frequency four one-proton doublets are observed in an ABCD spectral pattern characteristic of a phenolic ring flipping at a rate slow in the 600-MHz time scale; at 300 MHz, the resonances are too broad to be observed, indicative of a ring flipping at $\lesssim$300 Hz. Expected positions of the Tyr-V B and D doublets in the 300-MHz spectrum are indicated by arrows (A). The Tyr-I BC resonances appear broader at 600 MHz (B), which indicates a ring flipping rate $\lesssim$ 600 Hz (they generate a neat BB′ doublet at 300 MHz).

Strong NOEs are also elicited from signals at ~6.7 and ~5.3 ppm. Unfortunately, because of small shifts induced by the addition of the ligand, 6-aminohexanoic acid, we cannot be certain as to whether the signal at ~6.7 ppm represents the expected (Poulsen et al., 1980) Trp H7 doublet or whether the doublet in question is the one weak signal shown by the NOE experiment at ~6.6 ppm (Figure 5C). Since the procedure has as yet not been implemented on the indole NH resonance of the third tryptophan, the singlet–doublet–triplet connectivity among aromatic protons of Trp-II and Trp-III remains unassigned. It should be recalled that titration with BASA shifts both singlet 1 and the triplet at ~6.7 ppm (Figure 4), suggesting these two signals might arise from the same indole, namely, that of Trp-II (Llinás et al., 1983). On the other hand, praseodymium ions induce shifts that suggest it is singlet 7 that should be paired to the rest of the Trp-II aromatic spectrum (N. D. Pluck and R. J. P. Williams, unpublished observations). The correspondence of singlets 1 and 7 with the rest of the indole spectra requires future investigation.

Kringle 4 contains five tyrosyl residues and one phenylalanine. Of the tyrosyl aromatic signals, only four can be detected at 300 MHz (Figure 6). An expansion of the aromatic SECSY spectrum is depicted in Figure 6B to indicate connectivities within tyrosyl ortho–meta AA′XX′ two-doublet phenolic spin systems. Figure 6A also indicates a one-proton Phe^{64} [Phe^{63} in the numbering of Trexler et al. (1983)] triplet, well outlined at ~7.27 ppm. Unfortunately, the Phe^{64} (Phe^{63}) spectrum overlaps extensively with other intense signals, so that the connectivities for the Phe^{64}-coupled resonances indicated in Figure 6B (dashed lines) cannot be fully justified from the data. However, in the presence of BASA, at 55 °C, the degeneracy affecting the phenylalanyl resonances is partially removed (Figure 7). In the 1-D spectrum (Figure 7A), the H4 triplet and the lowest field component of the H3,5 triplet are resolved at 7.4 and 7.25 ppm, respectively. This situation is mirrored in the 2-D SECSY spectrum (Figure 7B), where the contours are sufficiently isolated to unequivocally characterize the phenylalanyl ring spin system. The absence of a central component in the triplets results from coherent transfer (Bain, 1980).

Kringle 4 aromatic spectra at 300 and 600 MHz, recorded at ~27 °C and pH* 7.2, are compared in Figure 8. At the lower field strength, the Tyr-I spectrum is of the AA′BB′ type owing to efficient ortho–ortho (H2,6) and meta–meta (H3,5)

chemical shift averaging, reflecting rapid ring flipping on the spectrometer frequency time scale. At 600 MHz, BB′ doublet degeneracy is maintained, but the resonance appears broadened, indicating an intermediate flipping regime on a shorter time scale. The magnetic field effect is even more dramatic for the Tyr-V resonances, undetected at 300 MHz but neatly visible as broad one-proton signals at 600 MHz (ABCD spectral pattern) meaning that the ring, immobile on the high-frequency time scale, flips at a rate $\lesssim$300 Hz. One must conclude that the Tyr-V ring is placed in a rather constrained structural niche.

Discussion

We have identified all three histidyl singlet pairs from their acid–base response and COSY spectra. From their pH dependence and H2 exchangeability, we were able to sort peaks from individual imidazole groups according to their H2 and H4 origin. From the spectra of ordinary, random-coil, histidyl residues (Markely, 1975) one would expect H2 and H4 resonances to appear at relative low- and high-field positions, respectively. Although this pattern holds for His-I and His-III, the singlet ordering is reversed for His-II, as shown above.

By comparing kringle 1 and kringle 4 spectra, we have tentatively assigned His-II to His[31], which is conserved in both kringles (Llinás et al., 1983). Thus, we confirm most histidine assignments given in previous papers (Hochschwender et al., 1983; Trexler et al., 1983; Llinás et al., 1983) with the exception of the singlet 8 Trp identification (Trexler et al., 1983), which should now be recognized as arising from the H2 of His-III (His-A). By default, singlet 7 must be assigned to the H2 resonance of an indole (Trexler et al., 1983), thus correcting the Trp identification proposed for singlet 2 (Llinás et al., 1983), which we now show is due to the H4 of His-III (His-A). The pH dependence of singlet 7 is not entirely unexpected in that a homologous resonance in the spectrum of bovine prothrombin fragment 2, which contains no histidine at all, also shows a similar acid–base response (M. P. Esnouf, N. D. Pluck, and R. J. P. Williams, unpublished results). Indeed, there is close similarity to the spectrum of bovine prothrombin fragment 2 where the tryptophan coupling schemes are extremely similar to those of Trp-I (Trp-A) and Trp-II (Trp-B) of kringle 4, as would be expected from homology arguments (M. P. Esnouf, N. D. Pluck, and R. J. P. Williams, unpublished results). In addition, further support is provided by the identification of kringle 1 of tryptophan H2 resonances to singlets 1 and 5 at chemical shift positions close to those of singlets 4 and 7 in the spectrum of kringle 4 (Llinás et al., 1983). Thus, we consider that the assignment of resonance 7 to a tryptophan H2 proton resonance remains reasonably well established.

The identification of the complete Trp-I (Trp-A) indole spectrum is reinforced by the observation that parallel to the acidic shift detected for singlet 4 at pH* $\lesssim$5 (Figure 2) we observe a titration of the paired Trp-I doublets and low-field triplet consistent with the grouping indicated in Figure 4. Interestingly, all these acid–base shifts disappear concurrently in the presence of BASA, suggesting that the ligand interferes with an interaction between the indole and an acidic side chain (e.g., from a glutamyl δ-COOH group) at the binding site.

The Phe[64] (Phe[63]) multiplets are difficult to recognize even at 45 °C, at which temperature they remain broad. It has been suggested (Trexler et al., 1983) that unresolved resonances at ~7.2 ppm arise from a phenylalanyl side chain. Here, we identify a one-proton triplet at ~7.2 ppm (Figure 6) as arising from that residue and unveil its complete aromatic spectrum in the BASA complex (Figure 7). With regards to tyrosyl

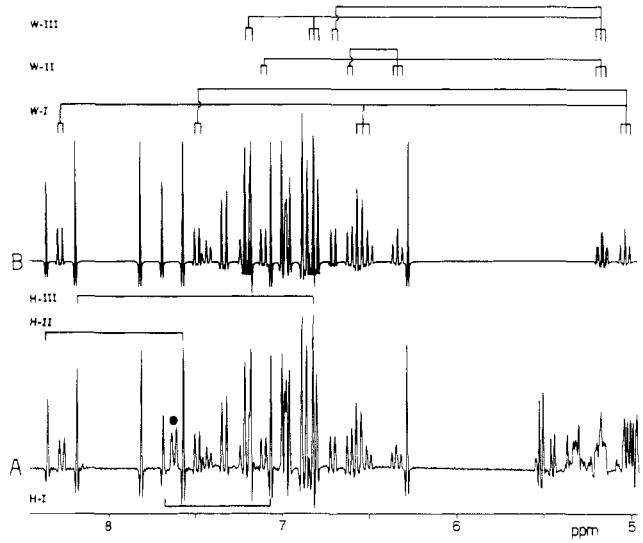

FIGURE 9: Aromatic ^{1}H NMR spectrum at 300 MHz of the kringle 4 in the presence of 2-fold excess BASA. (A) Experimental. The black dot denotes the doublet resonance from the BASA ring protons ortho to the acidic function. (B) Computer simulation of (A) accounting for the Gaussian resolution enhancement applied to the experimental spectrum. Between 5 and 5.5 ppm, only the three Trp triplets have been included in the simulation; their position was determined in a 2-D SECSY spectrum recorded under the same experimental conditions as spectrum A. Part of the 2-D spectrum is shown in Figure 7, which gives the experimental conditions. The BASA doublet (●) is excluded from (B). The individual Phe and Tyr spin systems of the kringle 4–BASA complex are described in Figure 7 and elsewhere (Llinás et al., 1983), respectively. His (H) and Trp (W) connectivities are indicated. Blackened resonances denote the Trp-III multiplets. Trp-II and Trp-III triplets overlap at ~5.20 ppm, and a Tyr-I doublet partially overlaps with a Trp-I triplet at 6.55 ppm.

spectra, the phenolic ortho–meta doublet pairs we identify coincide with those resported earlier (Trexler et al., 1983; Llinás et al., 1983) as follows: Tyr-I, Tyr-A; Tyr-II, Tyr-B; Tyr-III, Tyr-D; Tyr-IV, Tyr-C. Here, we complete the characterization of the kringle 4 tyrosyl spectrum by identifying the four one-proton phenol resonances of Tyr-V, which is totally immobilized on the 600-MHz spectrometer time-scale (Figure 8).

Because of the enhanced resolution of the BASA–kringle 4 complex spectrum (Hochschwender et al., 1983), it was selected for a complete aromatic spectral analysis. Figure 9 shows experimental (A) and computed (B) aromatic spectra at 300 MHz of kringle 4 in the presence of ~2:1 excess BASA at pH* 7.2, 55 °C. Since Tyr-V is not observed at 300 MHz (Figure 8A), it has been ignored in the simulation. The free ligand aromatic spectrum contributes an exchange-broadened component at ~7.1 ppm (not seen in the resolution-enhanced spectrum) and a doublet [indicated by a dot (●)] at ~7.65 ppm. Neglecting the ligand resonances, the simulation satisfactorily matches the experimental spectrum.

From previous studies it is known that aromatic residues are likely to be involved in the binding of lysine and analogous ω-amino acids and that they are important in forming the hydrophobic core that presumably governs folding of the polypeptide chain (Hochschwender & Laursen, 1981; De Marco et al., 1982; Hochschwender et al., 1983; Llinás et al., 1983; Trexler et al., 1983). In conjuction with aliphatic residues, such aromatic groups, in particular Trp[72] (Trp[71]), are thought to contribute a hydrophobic component to the lysine binding site while determining the kringles' ligand specificity. Therefore, aromatic residues ought to be viewed as fullfilling critical roles when characterizing the various physiological

functions of plasminogen in haemostasis, such as its attachment to fibrin, binding of α_2-antiplasmin, and complexation with the preactivation peptide (Collen, 1980; Thorsen et al., 1981; Sjöholm et al., 1973). From this viewpoint, the aromatic signal identification achieved in the present study affords a sound basis for attempting a structural characterization of the kringles' lysine binding sites by NMR experimentation. Such investigations are in progress in our laboratories and their results shall be reported in due course.

Indole {N*H*}–C*H* NOE experiments on the BASA complex of kringle 4 performed after this paper was accepted for publication (A. Motta, R. A. Laursen, and M. Llinás, unpublished results) demonstrate unambiguously that the Trp-II doublet at 6.65 ppm corresponds to singlet 7 at 7.80 ppm (Figure 9), thus confirming the Trp-II (Trp-B) pairing scheme proposed by N. Pluck and R. J. P. Williams on the basis of paramagnetic lanthanide ion titration experiments. This identification leads to assigning the Trp^{72} singlet 1 to Trp-III (Trp-C).

Registry No. His, 71-00-1; Trp, 73-22-3; Tyr, 60-18-4; Phe, 63-91-2; plasminogen, 9001-91-6.

References

Bain, A. D. (1980) *J. Magn. Reson. 39*, 335–342.

Bundi, A., & Wüthrich, K. (1979) *Biopolymers 18*, 285–297.

Castellino, F. J., Ploplis, V. A., Powell, J. R., & Strickland, D. K. (1981) *J. Biol. Chem. 256*, 4778–4782.

Collen, D. (1980) *Thromb. Haemostasis 43*, 77–89.

De Marco, A. (1977) *J. Magn. Reson. 26*, 527–528.

De Marco, A., Hochschwender, S. M., Laursen, R. A., & Llinás, M. (1982) *J. Biol. Chem. 257*, 12716–12721.

Ernst, R. E. (1966) *Adv. Magn. Reson. 2*, 1–135.

Ferrige, A. G., & Lindon, J. C. (1978) *J. Magn. Reson. 31*, 337–340.

Hochschwender, S. M., & Laursen, R. A. (1981) *J. Biol. Chem. 256*, 11172–11176.

Hochschwender, S. M., Laursen, R. A., De Marco, A., & Llinás, M. (1983) *Arch. Biochem. Biophys. 223*, 58–67.

King, G., & Wright, P. E. (1982) *Biochem. Biophys. Res. Commun. 106*, 559–565.

Llinás, M., De Marco, A., Hochschwender, S. M., & Laursen, R. A. (1983) *Eur. J. Biochem. 135*, 379–391.

Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70–80.

Olsson, G., Andersen, L., Lindquist, O., Sjölin, L., Magnusson, S., Petersen, T. E., & Sottrup-Jensen, L. (1982) *FEBS Lett. 145*, 317–322.

Poulsen, F. M., Hoch, J. C., & Dobson, C. M. (1980) *Biochemistry 19*, 2597–2607.

Sjöholm, I., Wiman, B., & Wallén, P. (1973) *Eur. J. Biochem. 39*, 471–479.

Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., & Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis 3*, 191–209.

Thorsen, S., Clemmensen, I., Sottrup-Jensen, L., & Magnusson, S. (1981) *Biochim. Biophys. Acta 668*, 377–381.

Trexler, M., & Patthy, L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2457–2461.

Trexler, M., Bányai, L., Patthy, L., Pluck, N. D., & Williams, R. J. P. (1983) *FEBS Lett. 154*, 311–318.

Saccharomyces cerevisiae Structural Cell Wall Mannoprotein†

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Received June 26, 1984

ABSTRACT: A novel mannoprotein fraction with an average molecular weight of 180 000 has been isolated from *Saccharomyces cerevisiae mnn9* mutant cell wall that was solubilized by β-glucanase digestion. The same material could be extracted from purified wall fragments with 1% sodium dodecyl sulfate. The protein component, 12% by weight, is rich in proline, whereas the carbohydrate, mainly mannose, is about evenly distributed between asparagine and hydroxyamino acids. Endoglucosaminidase H digestion of the isolated mannoprotein reduced its average molecular weight to 150 000, but the mannoprotein, while still embedded in the cell wall, was inaccessible to the enzyme. Biosynthesis and translocation of the mannoprotein were investigated by following incorporation of [^{3}H]proline into this fraction. In the presence of tunicamycin, both *mnn9* and wild-type X2180 cells made a mannoprotein fraction with an average molecular weight of 140 000, whereas in the absence of the glycosylation inhibitor, the *mnn9* mutant made material with a molecular weight of 180 000 and the mannoprotein made by wild-type cells was too large to penetrate the polyacrylamide gel. Although the cell wall mannoprotein was resistant to heat and proteolytic enzymes, attempts to isolate the carbohydrate-free component failed to yield any characteristic peptide material.

The yeast cell envelope has been postulated to contain at least three classes of mannoprotein: the *inducible hydrolytic enzymes*, such as external invertase and acid phosphatase, the *sexual agglutinins*, expressed on cells that are homozygous at the mating-type locus, and, lacking a better term, *structural mannoproteins* (Ballou, 1976). The external invertase of *Saccharomyces cerevisiae* (Gascón et al., 1968; Tarentino et al., 1974; Lehle et al., 1979) and the sexual agglutinins of *Hansenula wingei* (Crandall & Brock, 1968; Yen & Ballou, 1974; Burke et al., 1980) have been purified and analyzed in some detail, but little is known about the presumed structural mannoprotein other than what has been learned fortuitously from analyses performed on the total cell mannoprotein (Phaff, 1971; Ballou, 1976; Cohen et al., 1982).

†This work was supported by National Science Foundation Grant PCM 80-23388 and by U.S. Public Health Service Grant AI-12522. J.F. acknowledges support from the Deutsche Forschungsgemeinschaft.

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0006-2960/85/0424-0753$01.50/0