Kringle 4 from Human Plasminogen: A Proton Magnetic Resonance Study via Two-Dimensional Photochemically Induced Dynamic Nuclear Polarization Spectroscopy†

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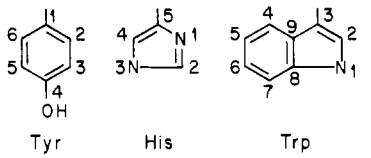
ABSTRACT: Two-dimensional (2D) proton magnetic resonance techniques used in conjunction with laser photochemically induced dynamic nuclear polarization (photo-CIDNP) spectroscopy have been applied to studying the kringle 4 domain from human plasminogen at 360 MHz. Out of 11 potential CIDNP-sensitive aromatic side chains, only 5 (His^3, Tyr^{41}, Tyr^{50}, Trp^{72}, and Tyr^{74}) appear to be accessible to 3-(carboxymethyl)lumiflavin, the dye used to photogenerate spin polarization. Of these, Trp^{72} and Tyr^{74} are known to be at, or near, the lysine-binding site. The spin–spin scalar (J) and phase-sensitive dipolar (Overhauser) connectivities in the 2D experiments yield absolute assignments for the aromatic signals stemming from the exposed tyrosyl and tryptophanyl rings. Moreover, a number of side-chain H$^\beta$ resonances can be identified and assigned to specific types of aromatic amino acid residues.

The heavy chain of plasmin contains a tandem array of five homologous domains named kringles (~10 000 Da each), of which kringle 1 (Lerch et al., 1980; De Marco et al., 1982), kringle 4 (Sottrup-Jensen et al., 1978; Lerch et al., 1980; Winn et al., 1980; Väli & Patthy, 1982), and probably kringles 2 and 5 (Trexler et al., 1982; Wallén, 1980) bind L-lysine and analogous ω-amino acids.

In previous NMR studies we (De Marco et al., 1982; Hochschwender et al., 1983; Llinás et al., 1983) and others (Trexler et al., 1983) have shown that the lysine-binding site is rich in aromatic residues; many of the aromatic side chains neighboring the binding site have also been identified (Llinás et al., 1985, De Marco et al., 1986). Among the latter, a key participant in ligand binding is the amino acid at site 72 (Hochschwender & Laursen, 1981; De Marco et al., 1982), which, in the case of kringle 4, is a Trp residue. Trp^{72} is known to carry a relatively exposed side chain since of all three Trp residues in kringle 4 it is the only one susceptible to chemical modification (Hochschwender & Laursen, 1981; Trexler et al., 1983). However, despite its functional relevance, the ^{1}H NMR spectrum of the Trp^{72} side chain has not yet been fully characterized. Thus, we still do not know which of the Trp^{72} aromatic doublets at 6.66 and at 7.05 ppm arises from the indole H4 or H7 protons (Llinás et al., 1985). Once either of these can be identified, the rest of the Trp^{72} aromatic spectrum becomes automatically assigned from the chemical shift correlated COSY[1] and SECSY connectivities (De Marco et al., 1985a; Ramesh et al., 1986). As discussed in the accompanying paper (Motta et al., 1986), selective Overhauser experiments in ^1H_2O do not lead to a clarification of this problem so that a different approach becomes necessary.

Chart I: CIDNP-Sensitive Aromatic Side Chains



Photochemically induced dynamic nuclear polarization (photo-CIDNP) experiments have long been established as a most useful tool when investigating surface residues of proteins by NMR spectroscopy (Kaptein, 1978, 1982) (see Chart I). However, despite the spectral simplification afforded by such experiments, overcrowding of resonances can still present a serious problem. This is of special concern when applied to proteins with several polarizable residues. For example, in hen egg white lysozyme it was found difficult to identify the spin system of one of the two polarizable Trp side chains because of resonance overlap in the aromatic region (Hore & Kaptein, 1983). Recently, 2D NMR methods have been applied to the photo-CIDNP experiment in order to overcome many of the problems encountered in the one-dimensional approach (Scheek et al., 1984, 1985; Redfield et al., 1985). The resolution gained by spreading the spectrum in a second dimension has the potential of better defining overlapping peaks, often of opposite CIDNP phase. Furthermore, COSY and NOESY connectivities in the photochemical experiment can simplify substantially the analysis of exposed aromatic spin systems. In addition, the enhancement of H$^\beta$ resonances and the am-

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[1] Abbreviations: CIDNP, chemically induced dynamic nuclear polarization; COSY, two-dimensional chemical shift correlated spectroscopy; FID, free induction decay; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect correlated spectroscopy; ppm, parts per million; pH*, glass electrode pH reading uncorrected for deuterium isotope effect; SECSY, two-dimensional spin-echo chemical shift correlated spectroscopy; 2D, two-dimensional.

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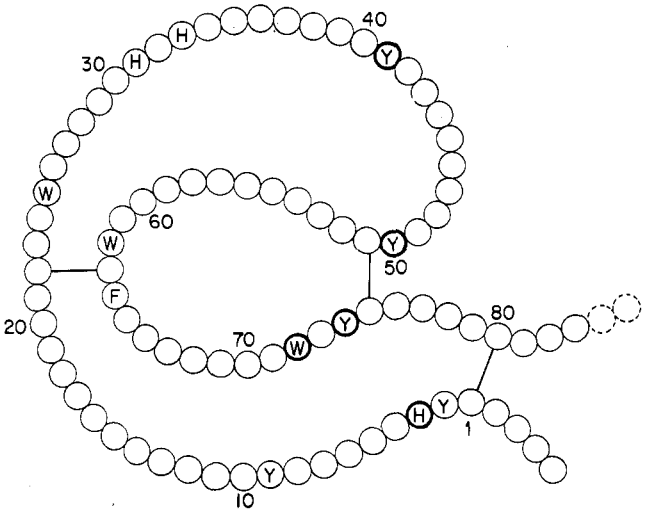

FIGURE 1: Human plasminogen kringle 4: outline of the structure. Aromatic residues are labeled according to the conventional one-letter code: H, histidine; F, phenylalanine; Y, tyrosine; W, Tryptophan. Experimentally found CIDNP-sensitive residues are circled with heavy trace. Two deletions have been inserted, at sites 35 and 59, to facilitate comparisons with other plasminogen kringles.

plification of dipolar interactions between these and the aromatic protons on the same side chain provide valuable assistance for unraveling standard NOESY spectra, which otherwise can be difficult to interpret because of unresolved cross-peaks.

This paper describes 2D-CIDNP COSY and NOESY experiments on ^2H_2O solutions of kringle 4 from human plasminogen (Figure 1). The information obtained relates both to the unveiling of exposed aromatic residues at the protein surface and to the establishing of absolute assignments for aromatic proton spin systems. Further, a number of H^β resonances can be selectively extracted out of the extremely crowded aliphatic spectral region, between 2.5 and 3.0 ppm, which is populated by unresolved broad resonances. In the case of kringle 4, Trp and His signals can be distinguished from those due to Tyr side chains by exploiting their different signs in the phase-sensitive presentation of the NOESY spectra. In particular, the experiments enabled the unambiguous identification of the Trp^{72} indole H4/H7 and H5/H6 resonance pairs, which manifest as doublets and triplets, respectively, in the conventional 1D experiment (De Marco et al., 1985a; Ramesh et al., 1986). It should be recalled that among the four types of aromatic side chains commonly found in proteins the phenylalanyl ring is the only one that is insensitive to the photo-CIDNP experiment (Kaptein, 1978, 1982). In the case of kringle 4, this means that one should not expect to observe direct CIDNP effects on Phe^{64}.

Experimental Procedures

Plasminogen was extracted from aged citrated human plasma, provided by the Central Blood Bank of Pittsburgh, and purified by affinity chromatography on lysine–Sepharose (Deutsch & Mertz, 1970). Kringle 4 was generated via porcine pancreatic elastase digestion of human plasminogen and purified by means of affinity chromatography on lysine–Sepharose and gel filtration on Sephadex G-75 (Sottrup-Jensen et al., 1978). For the NMR studies the protein was dissolved in ^2H_2O, pH* 7.2, 25 °C; the solutions were 1 m*M* in protein and 0.4 m*M* in flavin I [3-(carboxymethyl)lumiflavin]. The latter was a gift of Dr. F. Müller, Wageningen. The chemical shift scale is referred to internal sodium 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]propionate. Sample solutions were warmed at 28.5 °C for 5 h before the NMR measurements to exchange labile protons.

The 2D photo-CIDNP ^{1}H NMR spectra were recorded at 360 MHz on a Bruker HX-360 spectrometer, interfaced to an Aspect 2000 computer. To polarize the dye, the sample was irradiated at 488 nm with an argon ion laser (Spectra Physics 171). The NMR sample was mixed by a short increase of the spinning rate after each detection period (Scheek et al., 1984) in order to compensate the effects of dye exhaustion. Despite these precautions, the total number of experiments that can be performed with one sample is rather limited, and a number of expedients had to be used, such as positioning the carrier frequency at the low-field side of the aromatic region, so that quadrature detection in ν_1 is not necessary (Scheek et al., 1984). In order to excite only the neat photo-CIDNP response, a saturation train of 90° pulses was applied before each "light" scan (Schaeublin et al., 1977). Simultaneous acquisition of COSY and NOESY spectra was performed as previously described (Haasnoot et al., 1984; Gurevich et al., 1984). Sixty-four experiments were accumulated, and only one "light" FID was recorded at each t_1 value. To suppress background magnetization (including axial peaks), a "dark" spectrum was also recorded by interweaving 16 scans without laser irradiation at each t_1 value, and the difference spectrum (light minus dark) was calculated after appropriate scaling. The irradiation time was 0.2 s; the mixing time for the NOESY experiment was 0.2 s. The COSY matrix was 512 × 64 points in the absolute value presentation after 2D Fourier transformation; a sine bell window (De Marco & Wüthrich, 1976) was applied in both dimensions. The NOESY matrix was 1024 × 64 points; Fourier transformation was accomplished in the phase-sensitive mode after the application of a 3-Hz line broadening in ν_2 and of a cosine bell in ν_1. Data were processed on a VAX 11/750 computer. In order to maintain an acceptable digital resolution in the ν_1 dimension, despite the limited number of t_1 values, the sweep width along ν_1 was chosen to match the aromatic region. This causes multiple ν_1 folding, which in turn brings about the characteristic "zig-zag" appearance of the main diagonal in the 2D spectra shown in Figures 3 and 4 and, consequently, the need for more than one ppm scale along ν_1. This feature is not apparent in Figure 2 because of the narrower ppm range shown in both dimensions, as compared with Figures 3 and 4.

Results and Discussion

Kringle 4 contains a total of 12 aromatic residues (Figure 1): three His (at sites 3, 31, and 33), three Trp (at sites 25, 62, and 72), five Tyr (at sites 2, 9, 41, 50, and 74), and one Phe (Phe^{64}) (Sottrup-Jensen et al., 1978). The aromatic ^{1}H NMR spectrum of kringle 4 has been studied in detail and the spin system of individual residues identified and, except for Trp^{25} and Trp^{62} (Trp I and Trp II), assigned (De Marco et al., 1985a; Trexler et al., 1985; Ramesh et al., 1986).

Figure 2A shows the aromatic region of the 2D photo-CIDNP COSY spectrum of kringle 4 in ^2H_2O, after exchange deuteration of all labile hydrogen positions. The upper trace is a projection along ν_1 of the same spectrum, in which all peaks appear positive because of the absolute value presentation. A number of features are readily noticed in Figure 2A: (a) two intense signals at about 7.73 and 7.08 ppm that arise from the H2 and H4 protons of His I (His^3), indicating high exposure of the corresponding imidazole group; (b) two weaker signals near 6.6 ppm that stem from a doublet and a triplet of Trp III (Trp^{72}). The two latter signals are *not J* coupled (De Marco et al., 1985a; Ramesh et al., 1986); hence, in principle,

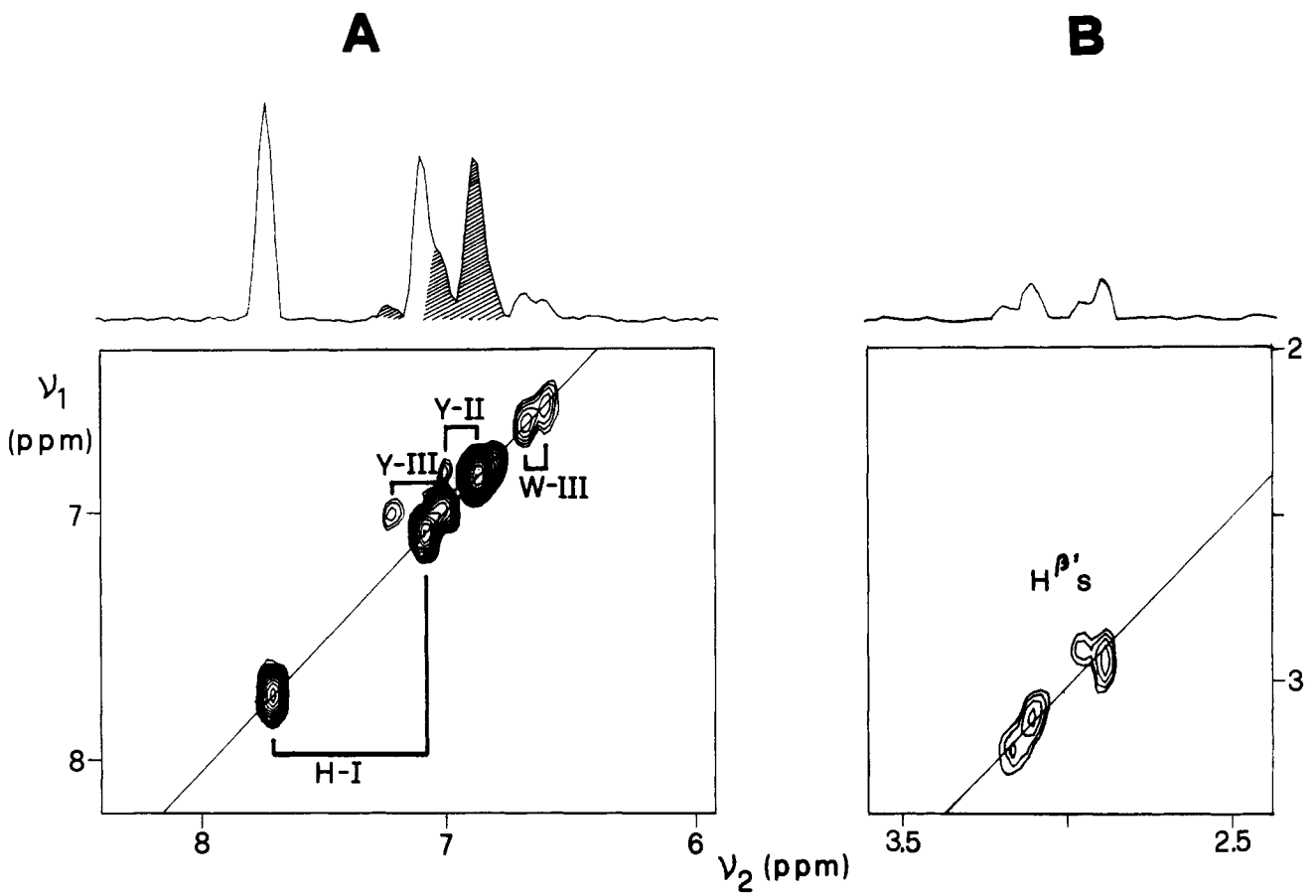

FIGURE 2: Photo-CIDNP COSY spectrum of kringle 4 from human plasminogen: (A) aromatic region; (B) aliphatic region. The projection along the ν_1 axis is shown on the top of each contour plot. Resonances are labeled according to the assignments discussed in the text. Sample conditions and technical details are described under Experimental Procedures.

they correspond either to H4 (doublet) and H6 (triplet) or to H5 (triplet) and H7 (doublet).

The absolute assignment of Trp resonances in protein NMR spectra is not straightforward. Provided that the indole NH signal is known and well isolated, the NOE from the Trp NH transition identifies the resonances from the neighbor H2 (singlet) and H7 (doublet) belonging to the same ring. Such an approach has been found successful for both the Trp I and Trp II (Trp^{25} and Trp^{62}) indole spectra, but for Trp III (Trp^{72}) the experiment could not be implemented (De Marco et al., 1985a; Motta et al., 1986). This is so because in ^2H_2O solutions the Trp^{72} indole NH exchanges fast and, therefore, the corresponding signal is not observable, while in ^1H_2O it appears to overlap with peptidyl amide resonances and cannot be resolved. Fortunately, for such exposed indole groups the photo-CIDNP experiment can be most informative as it primarily polarizes H4 and H6 resonances, which makes the analysis of Trp indole spectra unequivocal. Although the H2 singlet is also expected to yield an intense signal in the photo-CIDNP difference spectrum (Kaptein, 1982), the signal is apparently not self-evident in Figure 2A, where all remaining resonances arise from Tyr residues. However, under the conditions of our experiment the Trp^{72} (Trp III) H2 singlet is known to overlap with the H4 doublet from the same residue at ~6.66 ppm (Llinàs et al., 1985); hence, the signal at ~6.68 ppm most likely accounts for both protons, unresolved under the experimental conditions.

The Tyr resonances are shown as dashed areas in the COSY projection (Figure 2A). The signal at ~7.00 ppm corresponds to the H3,5 protons of Tyr^{41} (Tyr III), as these ring protons are known to experience the main enhancement in the photo-CIDNP experiment (Kaptein, 1982); a minor signal near 7.20 ppm is assigned to the H2,6 from the same Tyr^{41} ring as it arises from a cross-peak in the COSY spectrum. The intense signal at 6.88 ppm in the projection stems from the H3,5 protons of both Tyr^{50} (Tyr II) and Tyr^{74} (Tyr IV). However, in the COSY spectrum, only Tyr II exhibits H2,6–H3,5 cross-peak; the corresponding connectivity for Tyr IV can be observed in the NOESY contour plot (discussed below).

As is readily observed for Tyr^{41} and for Tyr^{50}, the H2,6–H3,5 cross-peak in the COSY spectrum is found only at one side of the main diagonal, i.e., at the same frequency ν_1 as the H3,5 signal, whereas the intensity of the H2,6 resonances is so weak that they do not show a peak on the diagonal, at least not above the threshold level chosen for plotting the contours illustrated in Figure 2A. This asymmetric appearance is a general characteristic of 2D photo-CIDNP spectra, caused by the asymmetry in the mixing process: only those magnetization components that are polarized during the preparation period (either by direct photopolarization or by cross-polarization from the dye) appear as diagonal peaks. Associated cross-peaks (at the same ν_1) are indicative of polarization transfer during the mixing period (Scheek et al., 1985).

Figure 2B shows the aliphatic region, between ~2.5 and ~3.5 ppm, of the 2D photo-CIDNP COSY spectrum. Only three signals are observed, at ~3 ppm, most likely arising from the polarized residues' H^β protons. Owing to a lack of phase sign information in the COSY experiment, these resonances cannot be assigned to specific types of amino acid residues; more definite conclusions can be drawn upon analysis of the NOESY data.

In contrast to the COSY results, the phase coherence of the multiplets intrinsic to the NOESY experiment makes it pos-

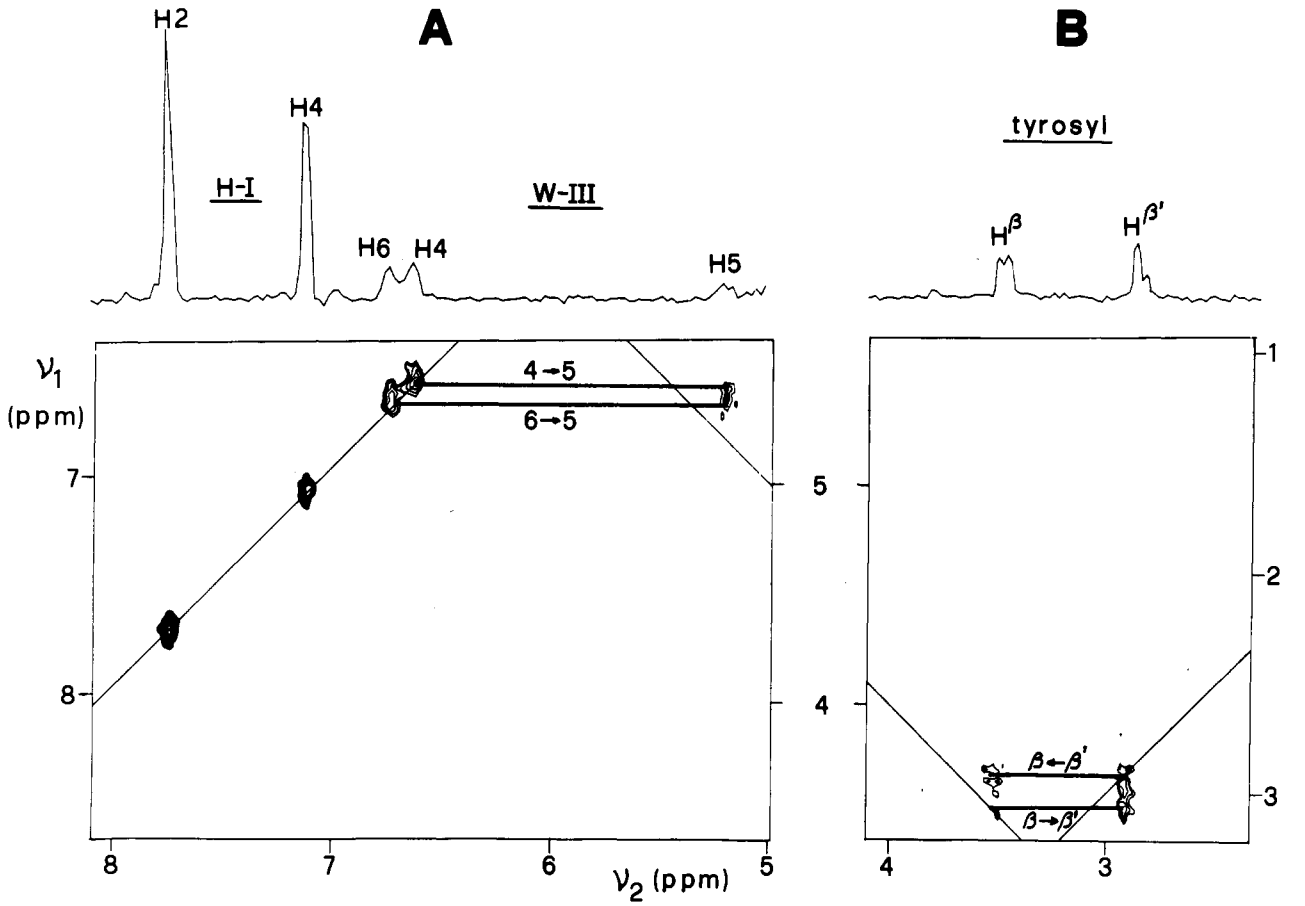

FIGURE 3: Photo-CIDNP phase-sensitive NOESY spectrum of kringle 4 from human plasminogen: positive levels. (A) Aromatic region; (B) aliphatic region. The projection along the ν_1 axis is shown on the top of each contour plot. Resonances are labeled according to the assignments discussed in the text. Sample conditions and technical details are described under Experimental Procedures.

sible to extract sign information by presenting the spectra in the pure absorption mode. Just as in the conventional 1D photo-CIDNP experiments, sign rules controlling the radical pair recombination reaction determine a pattern of signals and phases characteristic for each aromatic residue (Kaptein, 1978, 1982). In a fashion similar to that used for representing the COSY spectrum shown in Figure 2A, Figures 3A and 4A show contour plots of the aromatic region of the 2D photo-CIDNP NOESY spectrum of kringle 4 for positive and negative levels, respectively. The ν_1 projections have also been separated according to sign, which prevents accidental cancellation of peaks due to interference between positive and negative signals.

Positive signals in the aromatic photo-CIDNP spectrum arise from polarization of either Trp indole H2, H4, and H6 or His imidazole H2 and H4 ring protons; small positive signals may also stem from weak polarization of Tyr aromatic H2,6 transitions (Kaptein, 1978, 1982). In Figure 3A the projection of the positive levels shows two intense signals at 7.73 and 7.10 ppm, which have been assigned, as discussed above, to the H2 and the H4 singlets of His^3 (His I), respectively. The Trp^{72} (Trp III) signals near 6.7 ppm are better resolved in the NOESY spectrum than they were in the COSY experiment (Figure 2A). Moreover, an additional signal is now evident at ~5.2 ppm, i.e., at the H5 resonance position (De Marco et al., 1985a). This signal is due to intraresidue cross-polarization (Kaptein, 1982; Hore et al., 1982): the NOESY contour plot shows that the H5 peak is off-diagonal, with a width spanning a ν_1 range that covers both H4 and H6 chemical shifts, which suggests that the indole H5 undergoes polarization transfer from *both* vicinal protons, in agreement with the theoretical prediction (Hore et al., 1982).

Negative signals in the aromatic region of a photo-CIDNP spectrum can only arise from Tyr ring H3,5 protons (Kaptein, 1978, 1982). In Figure 4A the projection of the negative NOESY levels shows two signals at 7.00 and 6.88 ppm, already observed in the COSY spectrum, but now with improved resolution because of the phase-sensitive representation. The contour plot exhibits the H2,6–H3,5 connectivities for all the three Tyr rings, including that for Tyr^{74} (Tyr IV), missing in the COSY spectrum (Figure 2A).

A negative off-diagonal peak is detected at the ν_2 frequency of the Trp III H4 resonance and a ν_1 frequency that does not correspond to any of the aromatic resonances (Figure 4A). This peak stems from cross-polarization between H^{β} and aromatic proton resonances as the enhancement following direct polarization of the H^{β} resonances from Tyr, Trp, and His is opposite in sign to that experienced by the corresponding aromatic signals. Hence, Trp and His residues are expected to yield negative $H^{\beta,\beta'}$ lines while Tyr $H^{\beta,\beta'}$ proton resonances will appear as positive signals.

Figures 3B and 4B show the $4 \gtrsim \nu_2 \gtrsim 2$ ppm aliphatic region of the 2D photo-CIDNP NOESY experiment, in a display similar to that used for the COSY spectrum (Figure 2B). The projection of the positive levels (Figure 3B) shows two peaks that, when resolved in two dimensions, appear as four signals, two on the main diagonal and two off-diagonal, the latter indicating dipolar cross-relaxation between the two protons. This can readily be interpreted as due to a Tyr H^{β}–$H^{\beta'}$ spin system not identified in the COSY experiment. The projection of the negative levels shows two close signals (Figure 4B) near 3 ppm. When discussing the aromatic region, we noticed also the presence of a small, negative signal at $\nu_2 \sim 6.63$ ppm, i.e.,

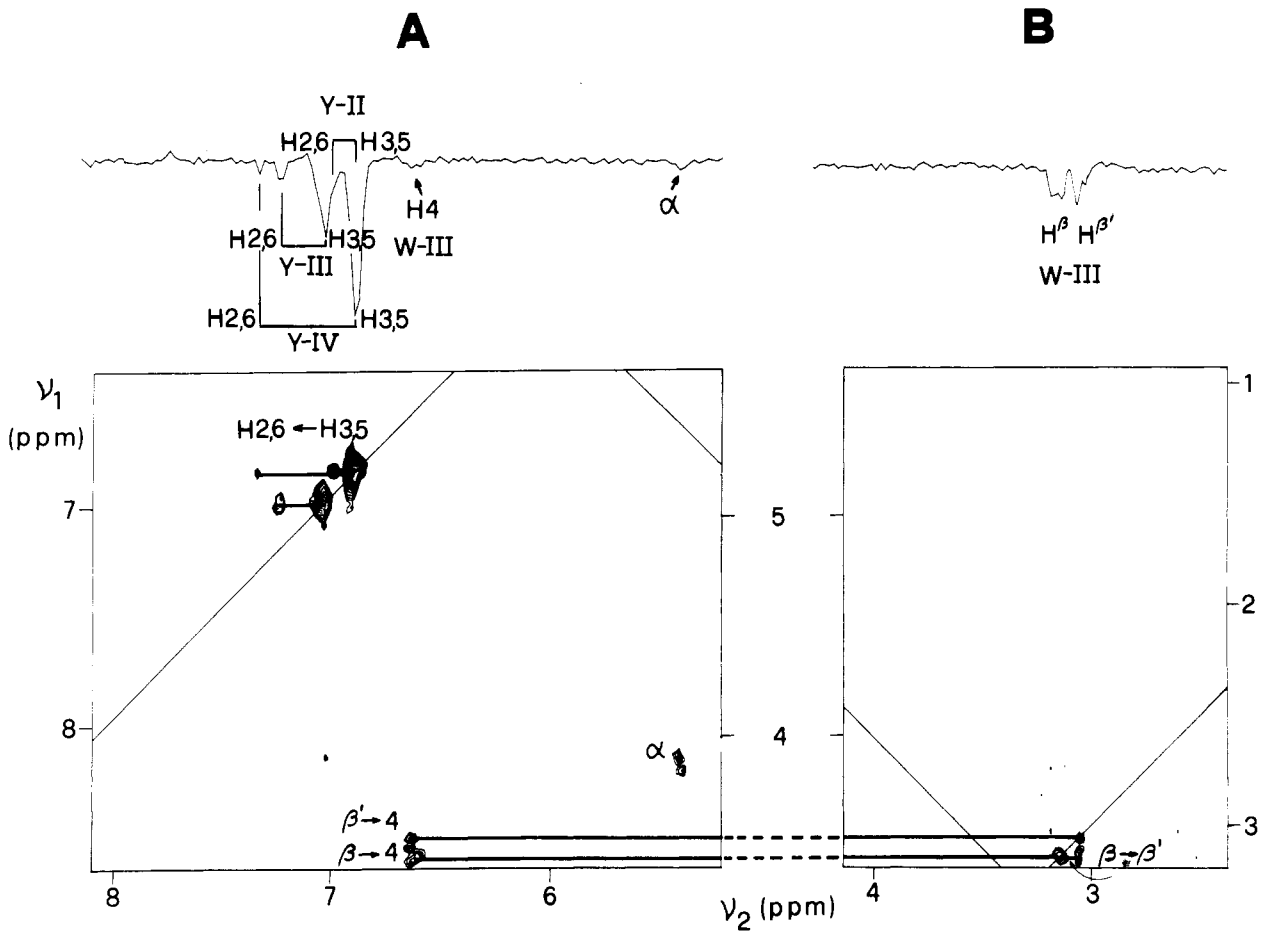

FIGURE 4: Photo-CIDNP phase-sensitive NOESY spectrum of kringle 4 from human plasminogen: negative levels. (A) Aromatic region; (B) aliphatic region. The projection along the ν_1 axis is shown on the top of each contour plot. Resonances are labeled according to the assignments discussed in the text. Sample conditions and technical details are described under Experimental Procedures.

at about the same position at which the Trp III H4 proton resonates in the COSY and in the NOESY photo-CIDNP spectra (positive levels). As indicated above, the negative signal is off-diagonal: by comparison of panels A and B of Figures 4, it is clear that the signal is due to cross-polarization from the H^{β}'s to the Trp III indole H4, which is known to occur with retention of sign (Kaptein, 1982). This assigns the spin system near 3 ppm to Trp^{72} (Trp III), rather than to His^{3} (His I).

Finally, a cross-peak at $\nu_2 \sim 5.4$ ppm (labeled "α" in Figure 4A) cannot be related to any of the observed aromatic or aliphatic signals. By exclusion, and taking into account its negative intensity, it is conceivable that it could result from strong cross-relaxation between the H^{β}'s and H^{α} of His^{3} (His I), where the diagonal peaks of the H^{β} protons, directly polarized, would be too weak to be detected in the contour plot.

Conclusions

The laser photo-CIDNP experiments on kringle 4 confirm the exposure of aromatic rings belonging to His^{3}, Tyr^{41}, Tyr^{50}, and Trp^{72}, as previously proposed on the basis of spin–spin relaxation (Llinás et al., 1983), pH titration (De Marco et al., 1985), Cu^{2+} binding (Trexler et al., 1983), and chemical modification (Hochschwender & Laursen, 1981; Trexler et al., 1983, 1985) experiments. Among the latter, Trp^{72} and Tyr^{41} are known to be at (Trp^{72}) or neighboring (Tyr^{41}) the lysine-binding site (Hochschwender & Laursen, 1981; Llinás et al., 1983, 1985; Trexler et al., 1983; De Marco et al., 1986). A novel finding is the exposure, as indicated by its CIDNP sensitivity, of Tyr^{74}, a residue that is also ligand sensitive (Llinás et al., 1985; De Marco et al., 1986). This is somewhat puzzling given that Tyr^{74} was found not to be susceptible to modification by tetranitromethane under conditions where Tyr^{41} and Tyr^{50} become nitrated (Trexler et al., 1985), especially taking into consideration that both the chemical modification and the photopolarization experiments proceed via the Tyr phenolic OH group. However, the Tyr^{74} side chain does not titrate below pH* ~11 (De Marco et al., 1986), which indicates a rather high pK_a for the phenolic group. This would interfere with the nitration reaction but probably not with the proton-abstraction free radical mechanism that has been postulated for the photochemical dye–side chain polarization transfer (Kaptein, 1982).

A main outcome of the CIDNP experiments is the information it provides for reaching an absolute assignment of aromatic Trp and Tyr resonances. Thus, from the CIDNP data we now know which of the phenolic ring doublets stem from the ortho (H2,6) and meta (H3,5) protons of Tyr^{41}, Tyr^{50}, and Tyr^{74}, information that otherwise would be difficult to obtain (Lecomte & Llinás, 1984; De Marco et al., 1985b). Furthermore, the CIDNP response of the Trp^{72} side chain leads to an unambiguous assignment of its indole spectrum, which, when combined with Overhauser experiments (De Marco et al., 1985a; Motta et al., 1986), brings to completion the analysis of the Trp aromatic spin systems found in kringle 4. Finally, the CIDNP data also provide valuable clues as to the location of specific aromatic H^{β} resonances within the aliphatic spectrum. Table I summarizes the pertinent information.

In a forthcoming paper (Manuscript in preparation) we shall report on the effects of antifibrinolytic ligands on the photo-CIDNP spectrum of kringle 4. In the interim, we can advance

Table I: Photo-CIDNP Assignments of Aromatic Resonances in the ^{1}H NMR Spectrum of Kringle 4

amino acid residue	proton type	δ (ppm)
His^3	H2	7.73
	H4	7.10
	H^α	5.41[a]
Trp^{72}	H4	6.63
	H5	5.20
	H6	6.73
	H^β	3.06, 3.14
Tyr^{41}	H2,6	7.20
	H3,5	7.02
Tyr^{50}	H2,6	6.98
	H3,5	6.88
Tyr^{74}	H2,6	7.34
	H3.5	6.88
Tyr	H^β	2.91, 3.51[b]

[a] Tentative assignment. [b] Not assigned to specific residues.

that in the presence of both *p*-benzylaminesulfonic acid and ϵ-aminocaproic acid the data confirm the His^3 H^α–H^β connectivity we now propose.

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

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