The solution structure of kringle 4

NMR studies on native and several chemically modified kringle 4 species of human plasminogen

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Kringle 4 of human plasminogen has been studied by NMR spectroscopy to define the solution structure of the kringle-fold and to characterize the ω-aminocarboxylic acid binding site. Aromatic and aliphatic resonances of the NMR spectrum have been identified with the aid of spin-decoupling and NOE procedures as well as pH-titration and metal ion probe studies. Comparison of the NMR spectrum of kringle 4 with the spectra of various kringle 4 species chemically modified at defined positions permitted the assignment of several resonances to specific residues in the kringle 4 sequence. The NOE studies revealed that Leu_{45} is in close proximity of the sequentially distant Trp_{25}/Trp_{61} residue pair, thus delineating a definite structural feature of the kringle-fold. The binding of 6-aminohexanoic acid to kringle 4 was shown to cause shifts in the resonances of several aromatic residues, including those of Trp_{71}, suggesting that several aromatic residues may be lining the ω-aminocarboxylic acid binding site. The binding of the ligand is competitive with the binding of lanthanide ions which reveal much detail of this site.

Human plasminogen *Kringle 4* *NMR spectroscopy*

1. INTRODUCTION

The non-protease part of plasminogen contains 5 closely homologous triple-loop structures, kringles [1], which play important roles in the interaction of plasminogen with fibrin, α_2-antiplasmin and ω-aminocarboxylic acids [2–4].

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Abbreviations: kringle 4, human plasminogen fragment corresponding to residues Val_{354}–Ala_{439} or Val_{354}–Val_{441} of plasminogen; in the case of kringles, numbering of amino acid residues starts at the first half-cystine residue of each kringle to facilitate comparison of the homologous kringles [1]; prothrombin fragment 1 and 2, fragments corresponding to residues 1–156 and 157–274 of prothrombin, respectively; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement

One of these structures, kringle 4 (fig.1), carrying an ω-aminocarboxylic acid binding site can be prepared by elastase digestion of plasminogen [1]. We have identified Asp_{56} and Arg_{70} on kringle 4 fragment as the residues involved in electrostatic binding of the ligand, thus proving that the ω-aminocarboxylic acid binding site is on the inner loop of the kringle [5].

Here, we have studied kringle 4 and several chemically modified kringle 4 derivatives by NMR spectroscopy to establish a solution structure for kringle 4 and to probe further into the nature of its ω-aminocarboxylic acid binding site. This paper is part of a series dealing with the NMR spectroscopy of kringles; e.g., on prothrombin kringles [6,7]. While this manuscript was in preparation a paper on the NMR spectra of kringle 1 of human plasminogen was published [8].

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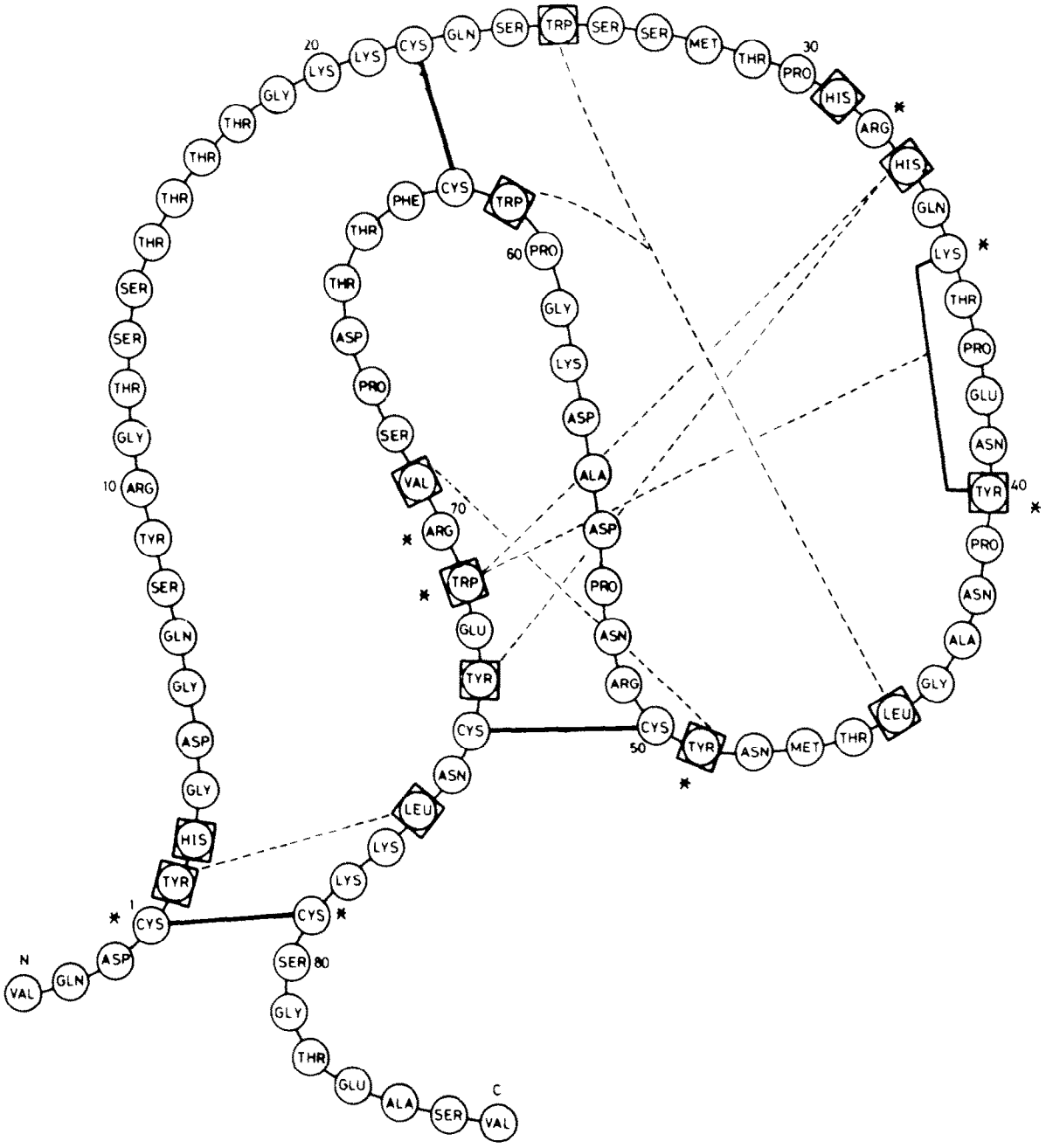

Fig.1. Structure of the kringle 4 fragment of human plasminogen [1]. The chemically modified kringle 4 derivatives used here were modified at the residue marked (*): (——) connecting Lys_{35} and Tyr_{40} represents the crosslink introduced between these residues; (□—□) residues whose resonances were identified in the NMR spectrum of kringle 4; (– – –) connect residues that were shown to be in close proximity to each other in the three-dimensional structure of the kringle.

2. MATERIALS AND METHODS

The isolation of the kringle 4 fragment and the preparation of the 1,2-cyclohexanedione-modified kringle 4 derivatives were performed as in [5]. The kringle 4 derivative in which Trp-71 is converted to

N-formylkynurenine was prepared by hydrogen peroxide oxidation as in [9]. The kringle 4 species, in which Tyr_{40} and Tyr_{40} + Tyr_{49} were converted to NO_2-tyrosines, were prepared by nitration with tetranitromethane [10]. Kringle 4, in which the Cys_1–Cys_{79} disulphide bridge is broken, was prepared by partial reduction of kringle 4 followed by alkylation with iodoacetate. This derivative retained affinity for lysine–Sepharose. Kringle 4 in which Tyr_{40} is crosslinked to Lys_{35} was prepared by reaction with 1,3-difluoro-4,6-dinitrobenzene [11]. Details of the modification procedures and the isolation and chemical characterization of the kringle 4 derivatives will be described elsewhere.

NMR spectra were obtained using a Bruker WH-300 and a 470 MHz spectrometer comprising an Oxford Instruments magnet and a Nicolet 1180 computer with a 293B pulse programmer. Spectra were routinely accumulated using 1 mM protein in 100 mM NaCl, 3 mM Tris in D_2O at 37°C. Dioxan was used throughout as an internal reference. The COSY experiment was performed at 47°C using a 5 mM protein sample in D_2O. pH of the solutions was measured using Pye-Ingold combined pH electrodes and the pH was changed using small amounts of molar DCl and NaOD.

3. RESULTS AND DISCUSSION

The results apply primarily to data obtained on kringle 4 of human plasminogen; where data obtained on the other kringles are referred to this is clearly indicated.

3.1. *Resonance assignments*

The aromatic and aliphatic regions of the 300 MHz NMR spectrum of kringle 4 are shown in fig.2,3. The assignment of the resonances to residue-type were made through the use of spin decoupling, two dimensional *J*-correlated (COSY), NOE and multiplet selection techniques. The spectrum is typical of a globular protein containing

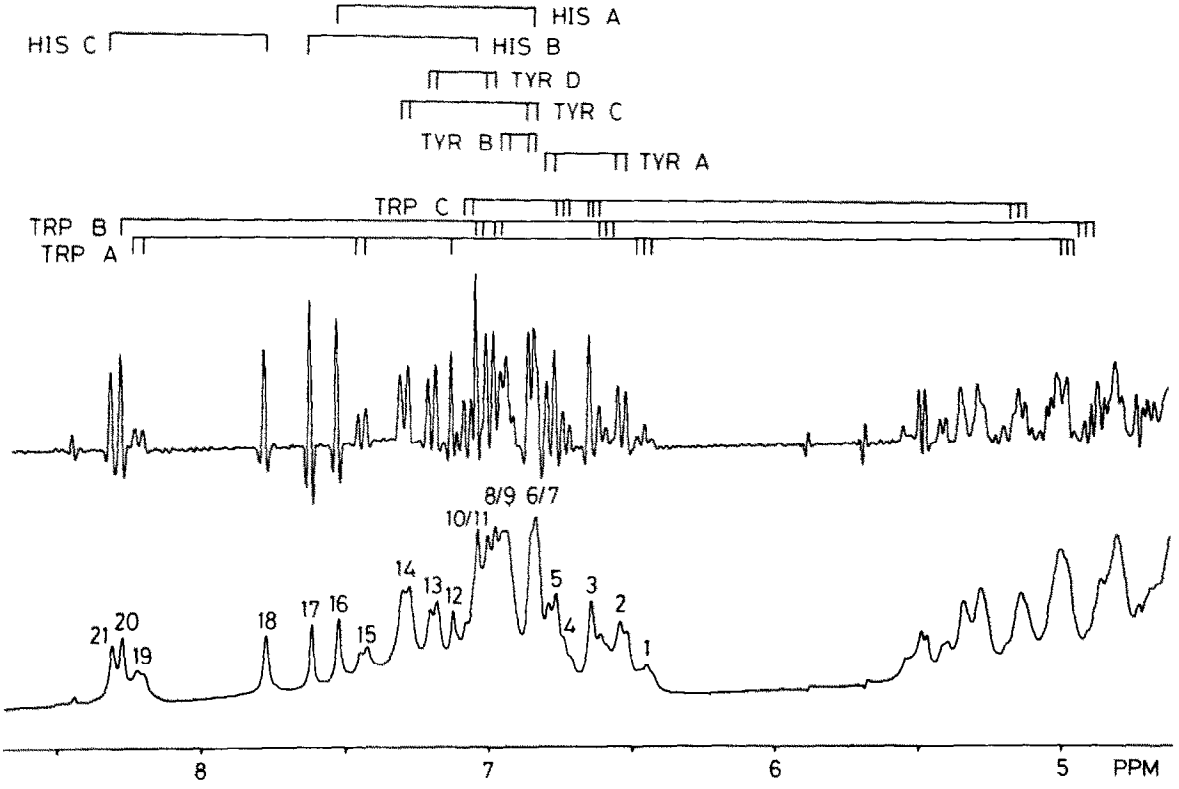

Fig.2. The aromatic region of the 300 MHz NMR spectrum of kringle 4 at 47°C (pH 8.0). The upper spectrum is the resolution enhanced form of the normal spectrum.

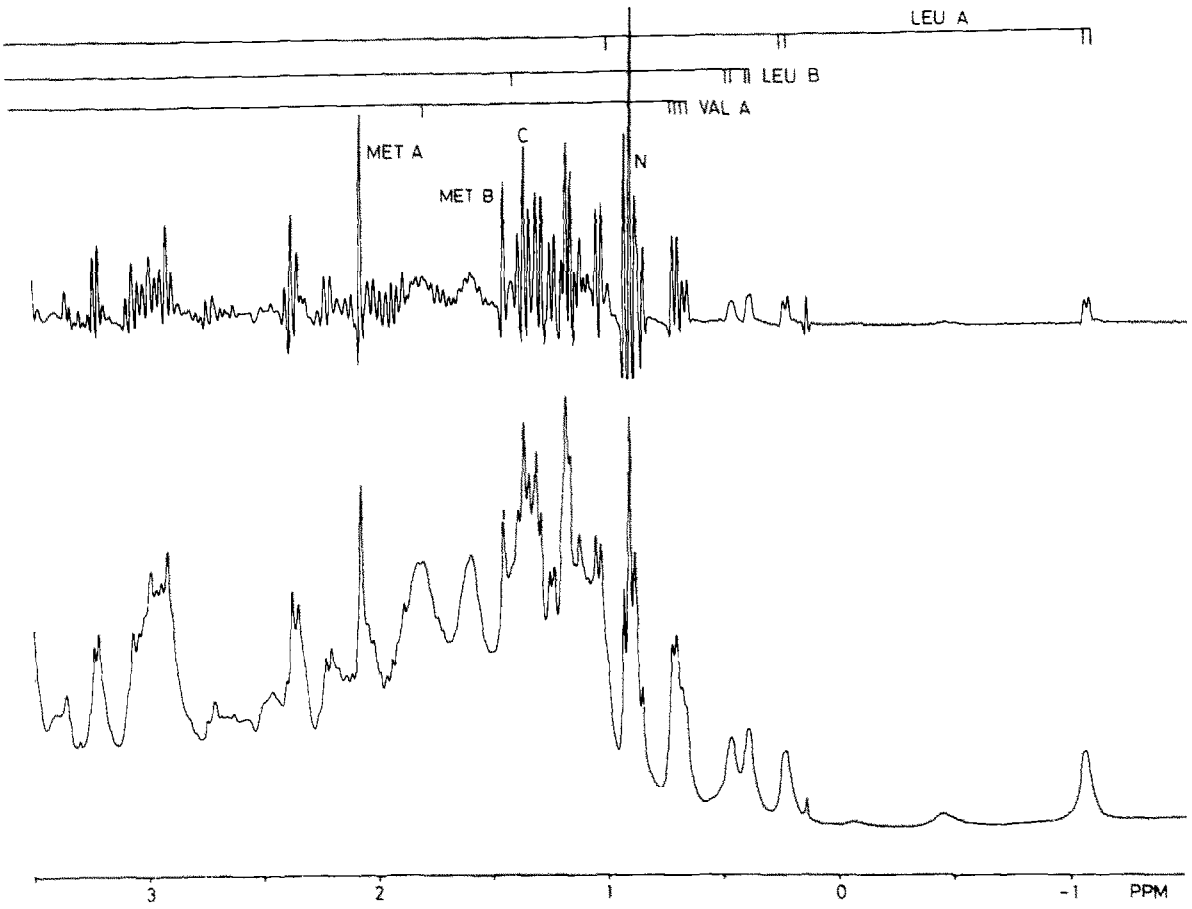

Fig.3. The aliphatic region of the 300 MHz NMR spectrum of kringle 4 at 47°C (pH 8.0). The upper spectrum is the resolution enhanced form of the normal spectrum. N and C indicate the sharp resonances arising from the N-terminal and C-terminal random-coil peptide regions.

resonances shifted upfield beyond 0.7 ppm, resonances from ~5.0–5.5 ppm, and a complex aromatic region of the spectrum well shifted from random-coil peptide chemical shift values. The amino- (N) and carboxy- (C) terminal residues extending beyond the kringle-structure appear to be in random coil since they give sharp lines indicative of considerable independent side-chain mobility compared to the resonances from side-chains within the kringle unit. For the labelling scheme of resonances see table 1.

Tryptophans: We have described a resonance in the spectrum of bovine prothrombin fragment 2 at 11.6 ppm that only disappears slowly on dissolving the protein in D_2O [6]. An analogous resonance appears in the same position and behaves similarly

Table 1

Summary of the residues assigned in the NMR spectrum of kringle 4

Trp A	Trp_{25}, Trp_{61}	Leu A	Leu_{45}
Trp B		Leu B	Leu_{76}
Trp C	Trp_{71}		
		Val A	Val_{69}
Tyr A	Tyr_2		
Tyr B	Tyr_{49}	Met A	Met_{28}[a]
Tyr C	Tyr_{73}[a]	Met B	Met_{47}[a]
Tyr D	Tyr_{40}		
His A	His_{31}[a]		
His B	His_3		
His C	His_{33}[a]		

[a] Tentative assignments

in the spectrum of kringle 4 and kringle 1 [8]. The unusual chemical shift position is strongly suggestive of an indole ring N–H proton resonance. NOE experiments in both proteins demonstrated spatial proximity to a one-proton singlet and one-proton doublet at very similar chemical shifts in both bovine prothrombin fragment 2 and kringle 4. Thus, these resonances are assigned to tryptophan A (C2 and C7) and at 7.12 ppm and 7.44 ppm, respectively, in kringle 4. Because only Trp_{25} and Trp_{61} are common to kringle 4, kringle 1 and bovine prothrombin fragment 2, Trp A must be assigned to either Trp_{25} or Trp_{61}.

The coupling scheme of Trp B is more tentative than that of Trp A in that two one-proton doublets and the most upfield one-proton triplet are not clearly visible as such since they lie amongst overlapping resonances. The one-proton singlet at 8.27 ppm is assigned as C2 of Trp B since it is the only remaining singlet attributable to a tryptophan after all the other singlets had been assigned. In addition, the chemical shift is very similar to that of a one-proton singlet in the bovine prothrombin fragment 2 spectra [6,7] which has only two tryptophan singlets (no histidine) in the aromatic region of the spectrum. Thus, this also identifies Trp B as the other tryptophan of the Trp_{25}/Trp_{61} pair clustered around the second disulphide bridge. The assignments are consistent with the data presented on kringle 1 [8].

While the singlet resonance of Trp A and B were present in the spectrum of the Trp_{71} oxidised kringle 4, the resonances assigned to the third Trp (Trp C) were absent. Thus, these resonances arise from the protons of Trp_{71}.

Histidines: pH titrations have demonstrated the C2 and C4 protons of each histidine – the C2 proton resonance having the larger chemical shift in each case. The NMR spectrum of the kringle 4 derivative in which Arg_{32} was modified with 1,2-cyclohexanedione was similar to that of native kringle 4, but the pH-dependence of the spectrum demonstrated a small change in the titration curves of two histidine resonances His A and C but that of the third histidine, His B, was unaltered. From these results it is reasonable to suggest that the histidine resonances affected by the modification of Arg_{32} originate from His_{31} and His_{33} adjacent to Arg_{32}, whereas the histidine residue unaffected is the sequentially distant His_3; i.e., His B. The assignment of His_3 is further supported by the results of Cu^{2+} titration experiments (see below). His C, one of the resonance pairs assigned to His_{31}/His_{33}, is noticeably altered in the kringle 4 derivative in which Lys_{35} is crosslinked to Tyr_{40}; this residue may therefore correspond to His_{33} rather than the sequentially more distant His_{31} (A).

Tyrosines: Nitration of Tyr_{40} removed the peaks of Tyr D from the spectrum. Three new peaks of the nitrotyrosine could be followed readily through a pH titration. Only small shifts were observed on other resonances. Nitration of tyrosine 40 and 49 removed the peaks of Tyr D and Tyr B and peaks from the nitrated tyrosines could again be followed through a pH titration. pH titration of the native kringle showed that these two tyrosines have the lowest pK_a-values consistent with their preferential modification. Tyr A peaks were assigned to Tyr_2 using the effect of Cu(II)-binding to the N-terminus (see below). The assignment was confirmed by showing that Tyr A reverted to a random-coil spectrum on breaking the -S–S- bridge $Cys_1–Cys_{79}$, while Tyr B, C and D were unaffected. The tentative assignments of the broad tyrosine resonances, not shown in fig.2 to Tyr_9 and the resonances of Tyr C to Tyr_{73} need to be confirmed since they are based on several pieces of circumstantial evidence.

Phenylalanines: There is only one phenylalanine in the spectrum but no resolved resonances are readily detected for it at 30°C. At 40°C resonances sharpen at around the random coil Phe position at 7.2 ppm. These are the only unassigned resonances in the aromatic region of the spectrum and we associated them with Phe_{63}. Their behaviour under a variety of conditions associates them with resonances of Tyr_9 which are also broad under most conditions.

α-CH: There are several α-CH resonances in the spectrum at 5.0–5.5 ppm. These resonances are now known to come from β-structures. Their assignments will be described in a later publication.

Leucines: There are only two leucines in kringle 4. One of these, Leu_{45}, is also present in prothrom-

bin fragments 1 and 2. The two 3-proton peaks (Leu A) shifted most upfield must be assigned to Leu_{45} since corresponding doublets are seen in the spectra of bovine prothrombin fragments 1 and 2 [6,7] and in kringle 1 [8]. The chemical shift of the proton coupled to both doublets is entirely consistent with leucine.

The other leucine, Leu_{76}, is assigned from COSY and NOE data showing a C–H proton coupled to the 3-proton doublets to be at 1.45 ppm again entirely consistent with the C–H proton of a leucine residue and not a valine. The assignment is confirmed by copper titrations (see below) and by the observation that breaking the Cys_1–Cys_{79} disulphide bridge disrupts the interaction of Leu_{76} (B) with Tyr_2 (A).

Valines: The N- and C-terminal valines are in random-coil peptide regions and their resonances are very sharp lines indicating significant sidechain mobility. The N-terminal valine methyl–proton resonances were clearly demonstrated from their pH behaviour.

The only valine present within the kringle-boundaries, Val_{69}, was identified using COSY and NOE experiments. These data showed the C–H resonance coupled to the two 3-proton doublets to be at 1.8 ppm in accord with an upfield shift on a valine sidechain.

Methionines: The two peaks labelled Met A and Met B are identified as such from the multiplet selection experiment showing them to be 3-proton singlets and therefore assignable to the methyl groups of Met_{28} and Met_{47}.

3.2. *Residue–residue interactions*

Nuclear Overhauser experiments were done to demonstrate inter-residue enhancements to detect residue–residue interactions that may have important structural implications. NOE's from the methyl groups of Leu_{45} were detected on the C2 and C7 resonances of Trp A. Enhancements have also been seen in these methyl groups and the C2 of Trp B as well as Tyr D. These results are striking in that Leu_{45} (A) near to the third disulphide bridge must be close to Trp_{25}/Trp_{61} clustered around the second disulphide bridge. (Analogous results have been obtained with bovine prothrombin fragment 2 and from the data published on kringle 1 we can deduce that this kringle has the same fold property.)

Further NOE's were detected between: Leu_{76} (B) and Tyr_2 (A); Val_{69} (A) and Tyr_{49} (B); the C4 of His_{33} (C) and Tyr_{73} (C); and the C2 of His_{33} (C) and Trp_{71} (C).

Crosslinking of Lys_{35} and Tyr_{40} with 1,3-difluoro-4,6-dinitrobenzene had noticeable effects on the NMR spectrum of kringle 4. The effect on the resonances of Tyr_{40} (D) is due to the direct effect of chemical modification, its influence on Leu_{45} (A) and His_{33} (C) reflects the proximity of the modified residues to these amino acids. The influence on the resonance of Met B cannot be interpreted easily since Met_{28} and Met_{47} are both equally distant from the modified peptide region (residues 35–40). The influence of the modification on the resonances of Trp_{71} (C), however, indicates that this residue may interact with the modified peptide region and/or the reagent introduced in this region.

The above interactions between various residues of kringle 4 are shown in fig.1 (– – –). We confirmed the structural connections by probe experiments.

Cu^{2+} titrations: Cu^{2+} were added to the protein at pH 7.5 and this produced extremely selective broadening of resonances in the spectrum. His_3 (B) was extremely sensitive, followed very closely by Tyr_2 (A), Leu_{76} (B) and interestingly the additional methyl group overlapping the downfield methyl of Val_{69} (A). The resonances of the N-terminus were also greatly broadened and as the titration was being performed the solution became mauve, typical of the biuret reaction. As seen with polypeptides [12] we interpret this to mean that Cu^{2+} coordinates with His_3 (B) and the amino-group of the N-terminus. The influence of Cu^{2+} bound to the N-terminus and His_3 (B) on a tyrosine residue was used to assign the resonance to Tyr_2 (A) (see *Tyrosines*). Furthermore we note how closely these data corroborate the NOE data linking Tyr_2 (A) and Leu_{76} (B) so closely in space.

3.3. *The ω-amino-carboxylic acid binding site*

Titration with praesodymium at pH 5.5 resulted in a number of sizeable shifts of various resonances. Adding a stoichiometric amount of 6-aminohexanoic acid to the praesodymium-kringle 4 sam-

ple resulted in many of these resonances returning to their ligand-bound chemical shifts, thus we have a very sensitive probe of the ω-aminocarboxylic acid binding site. The most notably affected resonances were those from Trp_{71} (C), Tyr_{40} (D), Leu_{45} (A) and the largest shift on the C4 of His_{33} (C), smaller shifts occurred on resonances from Trp_{25} (A) and Trp_{61} (B), Tyr_2 (A) and Tyr_{73} (C), Met B and Val_{69} (A). Residues unaffected included Leu_{76} (B) and Met A. Furthermore the Trp_{71} (C) and Val_{69} (A) shifts are upfield whereas the rest are downfield indicating that Trp_{71} (C) and Val_{69} (A) are in a spatially distinct region from the other residues about the lanthanide binding site. The lanthanide ion binds in fast exchange to the protein and in the presence of 6-aminohexanoic acid shifts additional resonances due to a second binding site. Ala_{84} is lanthanide shifted as are some other unidentified aliphatic peaks.

Addition of 6-aminohexanoic acid to kringle 4 at pH 8.0, causes a number of small shifts of resonances in the spectrum. Residues most affected include Trp_{71} (C) and Trp A, His_{33} (C), Tyr_{40} (D) and probably Tyr_{49} (B), although there are some other unexplained intensity changes in the region of the aromatic spectrum. Residues unaffected include His_3 (B) and His_{31} (A), Tyr_2 (A) and Tyr_{73} (C) and the majority of the aliphatic region of the spectrum, except for the downfield methyl peak of Leu_{45} which also shows a small shift. Neither methionine is affected.

The ligand binding site was shown in our earlier chemical modification studies to lie across the two strands of the inner loop of kringle 4, Arg_{70} and Asp_{56} being involved in electrostatic binding of the ligand's carboxylate and ammonium groups, respectively [5]. NMR studies on kringle 4 that have lost affinity for ligand as a result of the modification of Arg_{70} with 1,2-cyclohexanedione have shown that its spectrum is very similar to that of the normal protein confirming that modification of Arg_{70} destroys ligand affinity because Arg_{70} is directly involved in ligand binding and not because the modification disrupted the protein-fold around the binding site. This localization of the binding site is further supported by our finding that oxidation of Trp_{71} adjacent to the essential Arg_{70} also abolishes the ligand-affinity of kringle 4, in harmony with the result in [13] that modification of Trp_{71} with dimethyl-1,2-hydroxy-5-nitrobenzyl-1-sulfonium bromide results in loss of ligand affinity of kringle 4. On the basis of these results and those with lanthanides it is plausible to assume that the influence of ligand on the resonances of Trp_{71} (C) reflects the proximity of this residue to the ligand binding site. Since NOE experiments have shown that Trp_{71} (C) is also close to His_{33} (C) (see above) the influence of ligand on the resonances of His_{33} (C) is also explained by its proximity to the binding site. In addition to these residues, Trp A and two tyrosines affected by ligand binding, Tyr_{40} (D) and Tyr_{49} (B) must also be assumed to be relatively close to the binding site, thus the binding site is probably lined by a cluster of hydrophobic residues from the inner loop and the long right-hand loop. The role of the left-hand loop appears to be less significant since disruption of the Cys_1–Cys_{79} disulphide bridge does not affect ligand affinity.

We stress that the above observations hold out a real promise that the outline structure of kringles can be solved without reference to X-ray diffraction data.

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