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Three-Dimensional Structure of the Kringle Sequence: Structure of Prothrombin Fragment 1†

Chang H. Park and Alexander Tulinsky*
Department of Chemistry, Michigan State University, East Lansing, Michigan 48824
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ABSTRACT: The three-dimensional structure of bovine prothrombin fragment 1 has been solved at 2.8-Å resolution. The electron density clearly reveals four disulfide bridges along with more than 80% of the side chains completely in density, which correspond faithfully to the kringle sequence, its preceding 30 residues, and the dodecapeptide carboxy terminal; the polysaccharide and the first 35 residues of the amino terminal of fragment 1 are disordered or about 40% of the structure. The folding of the kringle sequence is based upon close disulfide van der Waals contacts between Cys-87–Cys-127 and Cys-115–Cys-139 (4.1 Å between midpoints of the bridges), two antiparallel strands of highly conserved (113–118, 124–129) β-structure, and the stacking of some conserved aromatic residues, all near the center of the folded structure. Moreover, the overall folding appears to be duplicated as a pair of stacked duplex loops with an antiparallel open loop. The overall shape of the kringle structure approximates an eccentric oblate ellipsoid of dimensions 11 × 28 × 30 Å. The residues immediately preceding the kringle are dominated by α-helical structure (Phe-41–Cys-48; Leu-56–Glu-63). Residues Phe-41–Trp-42 and Tyr-45, which are conserved in factor IX, factor X, protein C, and protein Z, form another aromatic stacked cluster while the Cys-48–Cys-61 disulfide loop corresponds to the well-known α/β structural unit. The dodecapeptide carboxy-terminal interkringle chain extends along the periphery of the kringle in its plane and forms a β-structure with the kringle-closing Ser-140–Val-143 tetrapeptide.

The production of the fibrin clot in blood coagulation arises from a cascade of activation reactions in which precursor glycoproenzyme molecules are converted to enzymes (Davie & Fujikawa, 1975). Moreover, the process is accomplished at greatly enhanced efficiency in the presence of membrane and cofactor enzymes, Ca^{2+} ions, and phospholipid surface (Mann, 1984). Blood coagulation reactions are thusly localized via complexation among cascade components at the site of the vascular injury. With time, cellular plasminogen activators convert plasminogen to plasmin, which ultimately dissolves the fibrin of the blood clot (Jackson & Nemerson, 1980).

The penultimate step of the blood coagulation cascade is the proteolytic conversion of the vitamin K dependent zymogen prothrombin or factor II (M_r 74000) to thrombin or factor IIa (M_r 37400). The two proteolytic cleavages that occur are catalyzed by another vitamin K dependent enzyme, factor Xa (M_r 45300) (Mann, 1976). A crucial requirement of this particular activation is the interaction of factor Xa with prothrombin substrate on a membrane surface in the presence of Ca^{2+} ions (Gitel et al., 1973; Henricksen & Jackson, 1975; Nelsestuen, 1976, 1984). This occurs under the direction of a membrane-bound enzyme, factor Va, composed of a light (74000) and heavy (94000) chain, which serves as the organizer in the assembly of the prothrombinase complex formed with factor Va, prothrombin, factor Xa, Ca^{2+} ions, and the phospholipid surface (Nesheim et al., 1980). The light chain of factor Va interacts with the membrane and with factor Xa while the heavy chain interacts with the prothrombin substrate. The net result of the assembly of the prothrombinase complex is to produce an enormous change in the overall catalytic rate of prothrombin activation at physiologically relevant concentrations, resulting in about a 3×10^5 fold increase in the rate of thrombin generation. The utilization of prothrombin as an efficient substrate is due partially to a conformational change in prothrombin accompanying Ca^{2+} ion binding, which is required for membrane interaction (Nelsestuen, 1976; Prendergast & Mann, 1977; Bloom & Mann, 1978; Tucker et al., 1983).

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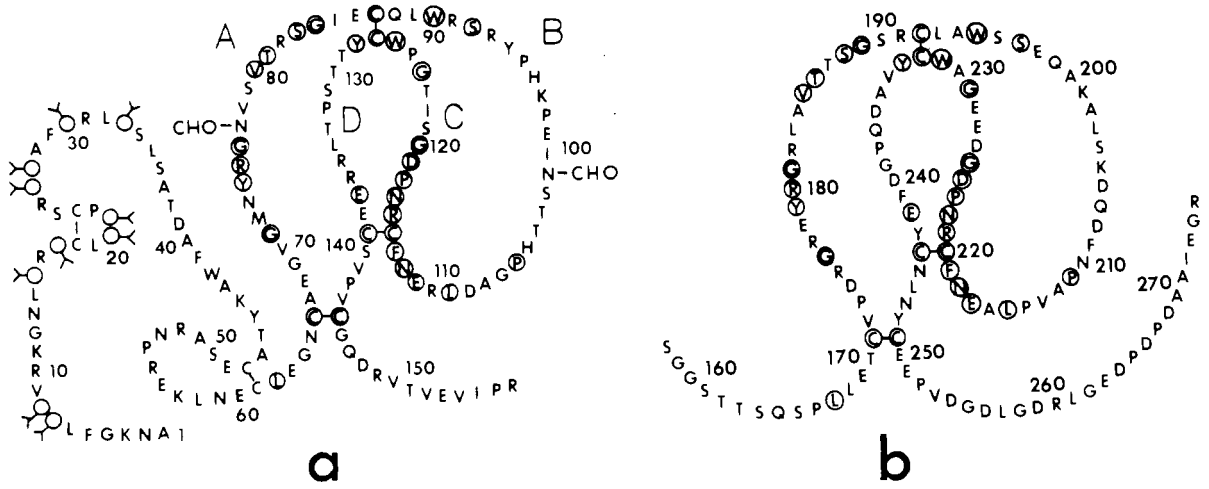

FIGURE 1: Sequences of bovine prothrombin fragment 1 (a) and fragment 2 (b): circles with Y's are γ-carboxyglutamic acid residues; CHO is for carbohydrate; residues identical in both sequences are circled; loop segments are designated A, B, C, and D.

Table I: Highly Conserved Kringle Residues[a]

	66	71	74–76	81	84	87	90	110	113–119	124–129	137	139
F1	C	G	YRG	T	G	C	W	L	NFCRNPD	GPWCYT	E	C
F2										GA V		
K1									Y	G	D	
K2			D						Y	- F		
K3									Y	A H		
K4									Y	G F		
K5									Y N	G	D	
UK				D					Y	- V	Q	
PA1				A					Y	- V		
PA2									Y	- HV		
FXII									A	- FV		

[a] Only exceptions to fragment 1 are noted: F1, fragment 1; F2, fragment 2; K1–K5, five kringles of plasminogen; UK, urokinase; PA1 and PA2, two kringles of tissue plasminogen activator; FXII, factor XII. Sequence number as in F1. Dashes at 124 denote deletions.

Prothrombin is composed of three structural domains commonly referred to as fragment 1, fragment 2, and prethrombin 2. The function of fragment 1 (Figure 1a) is phospholipid binding, that of fragment 2 (Figure 1b) is factor Va binding, while prethrombin 2 is the immediate precursor of thrombin. From the sequences in Figure 1 (Magnusson et al., 1975), it can be seen that duplication exists between most of the carboxy-terminal region of fragment 1 and the central part of fragment 2: specifically, between Cys-66–Cys-144 of fragment 1 and Cys-171–Cys-249 of fragment 2 where strikingly 31 of 78 residues (40%) are conserved. Such characteristic three-disulfide, triple-loop sequences of comparable sequence homology are also found among proteins involved in fibrinolysis, occurring singly, in pairs, or multiply, and they have become known as kringle sequences. Besides being duplicated in prothrombin, the kringle sequence repeats a spectacular 5 times in plasminogen (Magnusson et al., 1976), once in urokinase (Steffens et al., 1982; Gunzler et al., 1982), twice in tissue plasminogen activator (Pennica et al., 1983; Pohl et al., 1984; Ny et al., 1984), and once in factor XII (McMullen & Fujikawa, 1985). The high degree of homology displayed among kringle sequences (Figure 1 and Table I) has been the object of a number of comparisons and discussions related to residue sequence conservation and functional expression (Magnusson et al., 1976; Trexler & Patthy, 1983; Lucas et al., 1983); however, none have uncovered any general physiological functional relationships for a kringle sequence. A summary, for further consideration and discussion, of conservation shown by the 11 kringle sequences mentioned above is given in Table I.

Residues 1–45 of fragment 1 also display homology with the amino-terminal regions of factors IX and X of the blood coagulation cascade and proteins designated C and Z (Nelsestuen, 1984; Hojrup et al., 1985; Plutzky et al., 1986). All of the proteins contain γ-carboxyglutamic acid residues (Stenflo et al., 1974; Nelsestuen et al., 1974; Magnusson et al., 1974), which result from a postribosomal modification of glutamic acid residues by vitamin K (Esmon et al., 1975). A class of these relatively uncommon residues of prothrombin displays low cation specificity and additionally induces the conformational change essential for membrane binding (Nelsestuen, 1976; Prendergast & Mann, 1977). Partially γ-carboxylated prothrombin does not display the conformational change nor lipid binding and is not an efficient substrate in thrombin production (Malhotra et al., 1985). The remaining γ-carboxyglutamic residues of prothrombin function to bridge Ca^{2+} ions with negatively charged phosphate groups of a membrane surface to localize the protein on the phospholipid (Bloom & Mann, 1978).

Lastly, prothrombin is also a glycoprotein with two of its three polysaccharides attached to Asn-77 and Asn-101 of fragment 1 (Figure 1a), the two having a combined molecular weight of ~5000. The individual hexoses of the polysaccharides have been identified to be *N*-acetylglucosamine, *N*-acetylneuraminic acid, galactose, and mannose, and they have been sequenced by Mizuochi et al. (1979).

The X-ray crystallographic structure determination of a protein resident to blood coagulation has yet to be reported. This has been due to a variety of technical reasons, which range from insufficient amounts of material, purity, and stability for crystallographic purposes to failure to crystallize. Although prothrombin fragment 1 is adversely affected by its own set of technical problems (Tulinsky et al., 1985), nonetheless, it crystallizes under a number of different conditions (Aschaf-

fenburg et al., 1977; Hu-Kung & Tulinsky, 1980; Olsson et al., 1982). Moreover, it also displays some of the important activation and membrane-binding characteristics of prothrombin, rendering it pertinent to the mechanism of prothrombin activation.[1] In addition, fragment 1 contains a kringle sequence, the three-dimensional structure of which underlies the structure of a number of proteins involved in blood coagulation or fibrinolysis.

We have recently described an electron density of fragment 1 at 3.5-A resolution where long segments of polypeptide chain could be traced but could not be correlated with known sequence because there were many places where the density was not connected and/or more than one possibility existed as to which path to follow (Tulinsky et al., 1985). The difficulties encountered were partially due to the positional disordering of the polysaccharides of fragment 1, which comprise about 20% of the mass and electron content. Consequently, only a partial and fragmented chain folding was described (approximately 72% or 112 residues), which could not be matched with sequence information. We have been able to extend the resolution of the electron density of fragment 1 to 2.8 Å, which now clearly shows the position of four of the five disulfide bridges with three of them belonging to the kringle sequence. Moreover, all 112 aforementioned residues belong to the carboxy-terminal 121 residues of fragment 1, and as a result, we are able to report here the *kringle structure* or the first tracing of the three-dimensional folding of a kringle sequence. The carbohydrate moieties of fragment 1 remain disordered in the higher resolution map, which additionally revealed that the missing protein density of the 3.5-Å resolution structure corresponded to the first 35 residues of the amino terminal, which are likewise disordered. All of the general structural features of the 3.5-Å resolution work basically remain the same but now with sequence information assigned to them.

Materials and Methods

Bovine prothrombin fragment 1 has been kindly supplied to us throughout the course of these studies by Dr. G. L. Nelsestuen. The preparation of X-ray diffraction suitable tetragonal crystals of fragment 1 and heavy-atom isomorphous derivatives thereof for phase determination by the multiple isomorphous replacement (MIR) method has been described in detail previously (Hu-Kung & Tulinsky, 1980; Tulinsky et al., 1985).

The resolution of the electron density map of fragment 1 was extended from 3.5- to 2.8-Å resolution by using 3.5-Å resolution MIR phase angles and a single heavy-atom isomorphous derivative with 3.5–2.8-Å resolution data. The MIR phases were recalculated with the three best derivatives (Tulinsky et al., 1985) and then improved upon by employing Wang's (1985) solvent-flattening–phase-filtering program ISIR (average figure of merit $\langle m \rangle$ increased from 0.72 to 0.86 with $\langle \Delta\phi \rangle = 24°$), assuming 60% solvent based on mass-density considerations. These MIR phases were then used to calculate a solvent mask of a map that also included 3.5–2.8-Å resolution single isomorphous replacement (SIR) phase angles of a freshly prepared mercury acetate derivative, which was slightly different and displayed only two sites, 0.78 and 0.68 occupancy, 63 and 54 electrons, respectively.[2] This solvent-flattened map and subsequent ones were back-transformed, and new phase probabilities were computed in an iterative process. Masks were refreshed every 4–5 cycles of phase refinement, and convergence was attained with three solvent mask updates. The $\langle m \rangle$ increased from 0.69 for the MIR–SIR combined map to 0.81 for the final solvent-flattened 2.8-Å resolution map. The latter was calculated with 4858 native fragment 1 observations in contrast to only 2675 that were used to calculate the original 3.5-Å MIR map (Tulinsky et al., 1985).

Results and Discussion

General. The solvent-flattened 2.8-Å resolution electron density map of tetragonal crystals of fragment 1 possesses an enormous void region centered around $x = y = {}^1/_2$ and equivalent points with an average diameter of 35–40 Å running the entire length of the c axis (85 Å), closely similar to that observed in the 3.5-Å resolution MIR map (Tulinsky et al., 1985). The wall of this channel is composed of fragment 1 molecules arranged around 4_3 screw axes of the crystal.[3] It was shown previously at 3.5-Å resolution that deglycosylated fragment 1 is isomorphous with the glycoprotein and that the difference electron density map between the two possessed little density corresponding to the polysaccharides (Tulinsky et al., 1985). Therefore, it was concluded that the carbohydrate was disordered in the crystal. With the location of the saccharide-bearing residues of Asn-77 and Asn-101 in this work, it is now clear that the polysaccharides project into the cylindrical solvent channel and by virtue of their disorder contribute proportionately to the size of the void in the map.[4] In fact, the first two hexoses attached to Asn-77 and Asn-101 are conspicuous in the 2.8-Å maps.

During examination of the 2.8-Å resolution electron density corresponding to fragment 1 molecules, four disulfide bridges were evident along with more than 80% of the side chains completely in density (only a small number of Arg, Glu, and Lys were partially in or completely without density), attesting to the quality of the derivative and diffraction data, notwithstanding the tremendous carbohydrate disorder and other particular technical difficulties pertaining to the problem (Tulinsky et al., 1985). The foregoing along with the main chain density further indicated that the density corresponded faithfully to the kringle sequence, its preceding 30 residues containing a tetradecapeptide disulfide loop, and the dodecapeptide carboxy terminal. This suggested that the 35 amino-terminal residues might also be disordered (increasing the disorder to about 40% of the structure) or might have been proteolytically cleaved by a trace protease contaminant during the relatively long duration required for crystal growth (≈2 weeks). Although the latter alternative was not compatible with Ca^{2+} ion dependent quenching of intrinsic protein fluorescence of crystalline material (Tulinsky et al., 1985), nonetheless it prompted us to sequence the first five residues of crystals. These proved to be the expected ANKGF sequence, independently confirming the N-terminal disorder.

The Kringle Structure. The three-dimensional folding of the kringle sequence[5] appears to be based on a simple unifying

[1] Although Ca^{2+} ion binding to fragment 1 differs in some aspects from that of prothrombin, the former probably represents the Ca^{2+} ion binding to γ-carboxyglutamic acid residues of prothrombin.

[2] The mercury acetate derivative used in the 3.5-Å resolution MIR refinement had 78 and 39 electron occupancies along with two minor sites (16 and 7 electrons).

[3] Assuming the α-helical segments of fragment 1 are right-handed, the 2.8-Å resolution map revealed that the space group is $P4_32_12$ and not its enantiomorph.

[4] Mass-density considerations indicate about 60% solvent in tetragonal crystals whereas the electron density suggests even more or about 75% void space. The highly successful application of the solvent-flattening method for phase determination of fragment 1 was probably directly related to this highly favorable combination.

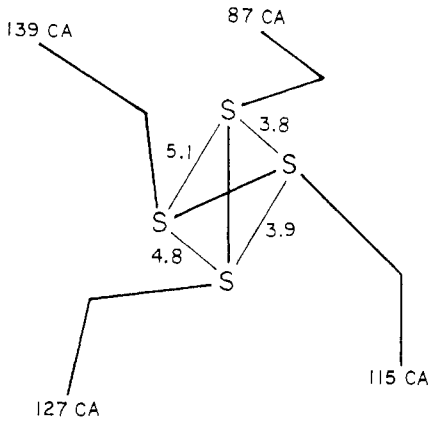

FIGURE 2: van der Waals contacts of Cys-87–Cys-127 and Cys-115–Cys-139: distances in angstroms; centers of disulfide bridges separated by 4.1 Å.

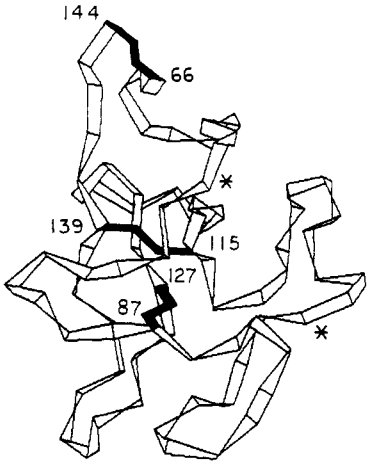

FIGURE 3: Schematic ribbon drawing of the folding conformation of the kringle structure: disulfides blackened and numbered; position of carbohydrate attachment denoted with an asterisk.

interaction: the sulfur atoms of disulfide bridges Cys-87–Cys-127 and Cys-115–Cys-139 make unusually close van der Waals contacts (Figure 2), which produce a sulfur cluster not far removed from the center of gravity of the kringle structure (Figure 3). The distance between the midpoints of the two disulfide bridges is only 4.1 Å, and two of the contacts border on the minimum possible (Figure 2). This intense interaction causes segments C and D of the inner loop of the kringle sequence (Figure 1a) to extend away from the disulfides as two antiparallel open-loop[6] structures, and since the disulfide bonds are roughly perpendicular to each other, the central loops are likewise (Figure 3). Thus, the internal loop of the kringle sequence (segments C and D of Figure 1a) becomes two unique loops in three-dimensional folding. Peptide segments Asn-113–Asp-119 and Gly-124–Thr-129 run antiparallel to each other as two β-strands and display most of the residue conservation of the kringle sequence, which is localized near disulfides Cys-87–Cys-127 and Cys-115–Cys-139 (Table I). The two loop structures appear to be of utmost importance to and serve as the nucleus of the kringle folded structure, which is consistent with the aerobic restoration of the foregoing disulfide bridges in reduced kringle 4 of plasminogen with

[5] In order to facilitate the description and discussion of the kringle structure, the segments of the three loop regions of the sequence have been designated A, B, C, and D (Figure 1).

[6] Open loops are here considered to be two continuous antiparallel chain segments; the combination of segments C and D, each of which forms a three-dimensional open loop, forms a closed loop.

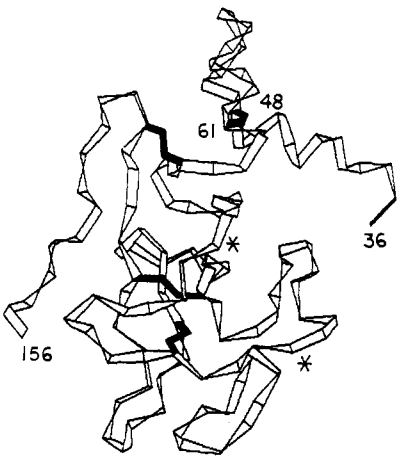

FIGURE 4: Schematic ribbon drawing of the folding conformation of prothrombin fragment 1: as in Figure 3 but with prekringle disulfide loop and amino and carboxy terminals numbered.

concomitant refolding (Trexler & Patthy, 1983). In fact, if the appropriate disulfide atoms of an approximately planar model of a kringle sequence (as in Figure 1) are brought upon one another in the proper way, the inner loop of the sequence (segments C and D) tends to spring into antiparallel loop conformations similar to those observed here.

Although segments A and B of the outer kringle sequence (Figure 1a) differ in length by seven peptide units, nonetheless they generally display a similar three-dimensional conformation, which also suggests a duplication in the folding (Figure 3); the principal difference between the conformations suggests the peptide insertion in B (Figure 3). The A and B segments each fold into two antiparallel open loops. The initial residues of segment A (near Cys-66–Cys-144) appear to mimic the folding of the antiparallel inner loop C and stack adjacent to it, while those of B (near Cys-87–Cys-127) copy D and stack with it (Figure 3). This gives rise to a pair of stacked duplex loop structures that are approximately related to each other by two independent 90° rotations. The apparently replicated loop of A might simply be the result of a chain buckling to accommodate the short Cys-139–Cys-144 tetrapeptide stretch closing the kringle structure (Figure 3). However, the B segment is not restrained by such constraints, yet the initial residues also fold in a manner strikingly similar to inner loop D, suggesting that the tetrapeptide segment cannot be the sole source of the formation of the copy loop of A. Thus, in summary, it can be said that the three-dimensional folding of the kringle sequence is, in general, orderly and possesses a somewhat intricately duplicated folding pattern. Moreover, the overall shape of the kringle structure resembles that of highly eccentric oblate ellipsoid of approximate dimensions 11 × 28 × 30 Å.

Amino-Terminal Region of Fragment 1 (Ala-1–Asn-65). The first 35 residues of the amino-terminal region of fragment 1, which also contain the 10 γ-carboxyglutamic acid residues, are like the carbohydrate of fragment 1 and are disordered in the crystal structure. The disorder poses an interesting question of functional relevance as to whether the region requires such freedom and flexibility to exhibit membrane binding. The disorder of the region is disappointing: because of it the exact nature of the γ-carboxyglutamic acid residues remains in doubt. However, the electron density is clear from Ser-36, and it extends continuously until it joins the kringle structure at Cys-66–Cys-144. This region notably contains the decapeptide of the amino-terminal 45 residues, which is homologous to a number of other vitamin K dependent plasma

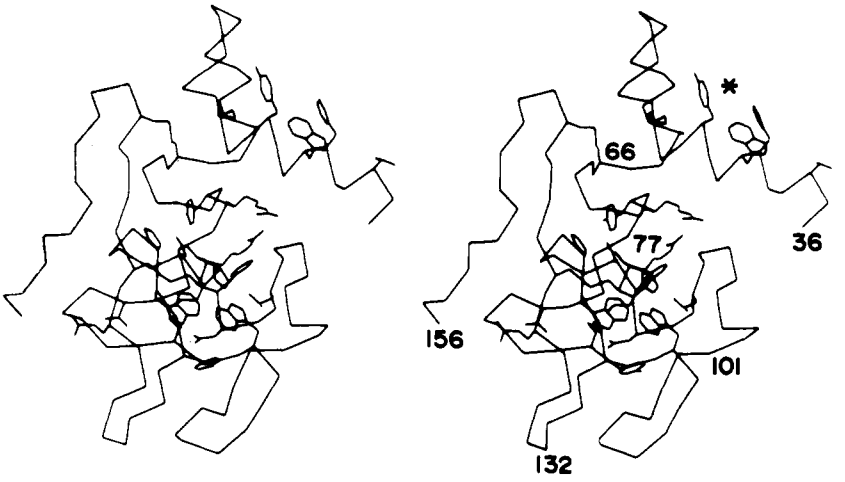

FIGURE 5: Stereoview of fragment 1 folding including conserved residues of Table I: main chain represented as CA; conserved Phe-41–Trp-42, Tyr-45 cluster denoted with an asterisk; Asn-77 and Asn-101, which carry carbohydrate, also noted.

proteins (Magnusson et al., 1975; Katayama et al., 1979; Enfield et al., 1980; Fernlund & Stenflo, 1980). It also contains the tetradecapeptide disulfide loop (Cys-48–Cys-61) of prothrombin and fragment 1, which is not unlike the more complicated disulfide loops of the growth factor structure (Gregory & Preston, 1977) found in other related proteins (McMullen & Fujikawa, 1985). This whole region of fragment 1 (Ser-36–Cys-66) is dominated by α-helical structure (Figure 4).

The Cys-48–Cys-61 disulfide loop of fragment 1 is preceded by about 2.5 turns of α-helix (Phe-41–Cys-48) (Figure 4) with residues Thr-38, Phe-41–Trp-42, and Tyr-45 of the region also being conserved in vitamin K dependent factor IX, factor X, protein C, and protein Z (Nelsestuen, 1984). The side chains of the conserved aromatic residues are stacked into an aromatic ring cluster (Figure 5), which has long been known to be an important stabilizing interaction pervading protein structures (Tulinsky et al., 1973). Since the aromatic residues are conserved in factor IX, factor X, protein C, and protein Z, a similar type of clustering can be expected to pertain in these molecules. A most unusual and conspicuous aspect of the cluster is that it resides on the surface of the fragment 1 molecule. Therefore, unless the ring cluster is covered in prothrombin, it will be starkly exposed to solvent, which raises alternatives to the stabilization–interaction function. In such a case, the cluster could serve as a recognition site of a receptor, enter into interaction with other aromatic clusters, or perform in some other useful functional context.

The closed Cys-48–Cys-61 disulfide loop of fragment 1, and presumably of prothrombin, is composed of a strand of extended chain that doubles back on itself at Pro-54 as two turns of α-helix (Leu-56–Glu-63) (Figure 4), producing the well-known α/β secondary structural unit. This α/β disulfide loop is approximately perpendicular to the preceding α-helix (Figure 4), and both lie in the plane of the kringle structure.

Interkringle Region. The dodecapeptide carboxy terminal of fragment 1 is about half of the interkringle region of prothrombin (Figure 1). The folded peptide makes a sharp turn at Gly-145 and then meanders in the plane of the semimajor axes of the kringle along its periphery roughly parallel to Ser-140–Val-143. This produces an antiparallel β-structural interaction for Asp-147–Thr-150 (Figure 4). The penultimate residue of the peptide is Pro-155, and since the second and third residues of fragment 2 are Gly-158–Gly-159, another sharp turn in chain path is more than likely in prothrombin. This could be part of a positioning mechanism by which proper stereochemistry is achieved for kringle–kringle interaction in the intact zymogen.

Residue Conservation and the Kringle Structure. From Table I, it is clear that the most significant conservation of kringle sequences occurs at Asn-113–Asp-119 and Gly-124–Thr-129, so it is not surprising that the two peptide regions are located adjacent and run antiparallel to each other near the center of the kringle structure as two strands of β-structure (Figure 5). In tandem with the disulfide cluster, they appear to form the basis of and underlie the folding of the kringle sequence. The Asn-113–Asp-119 sequence is practically conserved absolutely whereas the Gly-124–Thr-129 sequence exhibits a restricted flexibility. Although positions 128 and 129 display considerable variability (Table I), the aromaticity of the former is undeniable as is the stereochemical size and shape of the latter. Moreover, the turn at Gly-124–Pro-125 (inner loop C) is particularly noteworthy since it is so sharp (Figures 3 and 5) that it is difficult to fit the electron density with two undistorted residues. It can be seen from Table I that a deletion conveniently occurs at 124 in five kringle sequences and that a compensation occurs in prothrombin fragment 2 with the substitution of a Gly-Ala sequence; both suggest relief to a less bulky spatial arrangement.

It has already been noted that residue conservation in the amino-terminal segment of fragment 1 results in well-known aromatic ring clustering. The same pertains to some of the conserved aromatic residues of the kringle sequence. These are additionally and importantly located adjacent to the crucial disulfide cluster of the kringle structure (Figure 5). The particular residues are Trp-90, Trp-126, and Tyr-128, with Pro-118 enhancing the continuity of the ring stacking.[7] Thus, the *nucleus* of the unique chain folding of the kringle sequence appears to possess three major components: (1) two antiparallel strands of highly conserved β-structure, (2) extremely close van der Waals approaches of a pair of disulfide bridges, and (3) an aromatic stacked cluster (Figure 5).

Although the remainder of the conserved residues also tend to locate in the interior of the kringle structure, their role appears to be much more specialized and not always obvious. For instance, Gly and Pro residues usually serve to effect sharp turns in the main chain, and the stereotype of positions 114, 128, and 137 is quite clear. However, other residues are camouflaged by the intricacies of the tertiary structure and require more study for better understanding.

[7] Also noted in other structures (Tulinsky et al., 1973).

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Registry No. Prothrombin fragment 1, 72270-84-9.

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