The β Chain of Chicken Fibrinogen Contains an Atypical Thrombin Cleavage Site†,‡

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ABSTRACT: A cDNA corresponding to almost the entire coding region of the mRNA for the β chain of chicken fibrinogen was sequenced. At the protein level, significant homology to the β subunits of other vertebrate fibrinogens was found, with the highest degree of amino acid identity localized in the C-terminal region. In general, features conserved in the fibrinogens from other species also characterize the chicken sequence, including the cysteine motifs bordering an α-helical permissive region of fixed length and a single glycosylation site in the C-terminal region. However, the site of thrombin-catalyzed cleavage, which in other species consists of an Arg–Gly peptide bond, is instead an Arg–Ala bond in the chicken β chain. The Ala was confirmed directly from a sequencing analysis of the purified β chain of chicken fibrin. This finding may explain the observed slow clotting time of chicken fibrinogen relative to that of other species.

The central event in blood clot formation is the conversion of soluble fibrinogen—an oligomeric plasma protein composed of two sets of α, β, and γ subunits—into an insoluble fibrin clot. The limited protease thrombin cleaves the α and β chains of fibrinogen by specifically removing small N-terminal fragments (designated fibrinopeptide A and fibrinopeptide B, respectively), yielding fibrin monomers that polymerize to form the meshwork of a fibrin matrix [reviewed in Doolittle (1984)].

The mechanism of thrombin action and the kinetics of fibrinopeptide release have been studied in considerable detail (Blombäck et al., 1978; Nossel et al., 1983; Scheraga, 1983, 1986; Lewis et al., 1985). In species as diverse as human, bovine, dog, lamprey, and apparently also chicken, protein sequencing has shown that thrombin cleavage occurs at four specific Arg–Gly peptide bonds (one in each of the two α and β chains), generally situated within 20 amino acids from the N terminus (Iwanaga et al., 1969; Birken et al., 1975; Cottrell & Doolittle, 1976; Murano et al., 1977; Martinelli et al., 1979). In the case of human and lamprey fibrinogens, this result has been confirmed by cDNA sequence analysis (Rixon et al., 1983; Chung et al., 1983; Wang et al., 1989). The first avian fibrinogen subunit cDNA sequenced, that of the chicken α chain (Weissbach & Grieninger, 1990), also supports this pattern; however, analysis of the chicken β-chain cDNA and protein sequences, reported here, demonstrates the presence of an atypical thrombin cleavage site.

Materials and Methods

Reagents. Sequenase Version 2.0 sequencing kit and Gene 32 single-strand DNA binding protein were obtained from U.S. Biochemicals (Cleveland, OH). Reagents for immunoscreening a λgt11 cDNA expression library and the plasmid vector pGEM-3Z were from Promega (Madison, WI). Restriction enzymes were purchased from Gibco BRL (Gaithersburg, MD). Human thrombin was obtained from the Department of Blood Coagulation Research, Karolinska Institute, Stockholm, Sweden.

Cloning and DNA Sequencing. A cDNA library was constructed in the expression vector λgt11 by Clontech (Palo Alto, CA) by use of poly(A)-containing RNA from rooster liver supplied by this laboratory. Standard techniques for screening the library with either an antibody or an oligonucleotide probe were employed (Sambrook et al., 1989; Berger & Kimmel, 1987). Putative fibrinogen β-chain inserts were subcloned into the plasmid vector pGEM-3Z and sequenced by the dideoxy chain-termination method (Sanger et al., 1977) with use of Sequenase (Tabor & Richardson, 1987). Gene 32 protein was added to the sequencing reactions to prevent renaturation of denatured plasmid templates (Kaspar et al., 1989). Multiple alignments of protein sequences were performed by use of GENALIGN, based on the algorithm developed by Martinez (1988), and adjusted by visual inspection.

Fibrin Subunit Isolation and Amino Acid Sequencing. Isolation of the purified β chain of chicken fibrin was based on the procedure of Procyk and Blombäck (1990) for purifying the chains of human fibrin. Plasma was obtained from a 3.5-month-old rooster by bleeding in the presence of heparin to prevent clotting. Cellular debris was removed by centrifugation and the supernatant brought to 30% (v/v) ammonium sulfate saturation. The precipitate was pelleted, washed with a solution of 50 mM Tris,[1] pH 7.4, 100 mM NaCl, and 1 mM EDTA containing 30% ammonium sulfate, and resuspended in the same buffer containing Trasylol (5 kallikrein inhibitory units/mL) but without ammonium sulfate. The suspension was dialyzed against a solution of 50 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA, and human thrombin (10 units/mL) was added. The fibrin clot was allowed to form over a period of 60 min and then rolled up to remove any remaining liquid and dissolved in 75 mM Tris, pH 7.4, 8 M guanidine hydrochloride. The fibrin was completely reduced by the addition of dithiothreitol to a final concentration of 45 mM and incubated at 37 °C for 45 min. Free thiols were blocked by incubation with 4-vinylpyridine (88 mM final concentration) at 37 °C for 60 min. The reduced fibrin was dialyzed against FPLC starting buffer, consisting of 10% acetonitrile–0.2% trifluoroacetic acid, and the individual subunits were separated on an RPC PRO 5/10 column (Pharmacia, Piscataway, NJ) with a 30–42% acetonitrile gradient in 0.2% trifluoroacetic acid solution. The three distinct protein peaks observed in the gradient elution profile,

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[1] Abbreviations: FPLC, fast protein liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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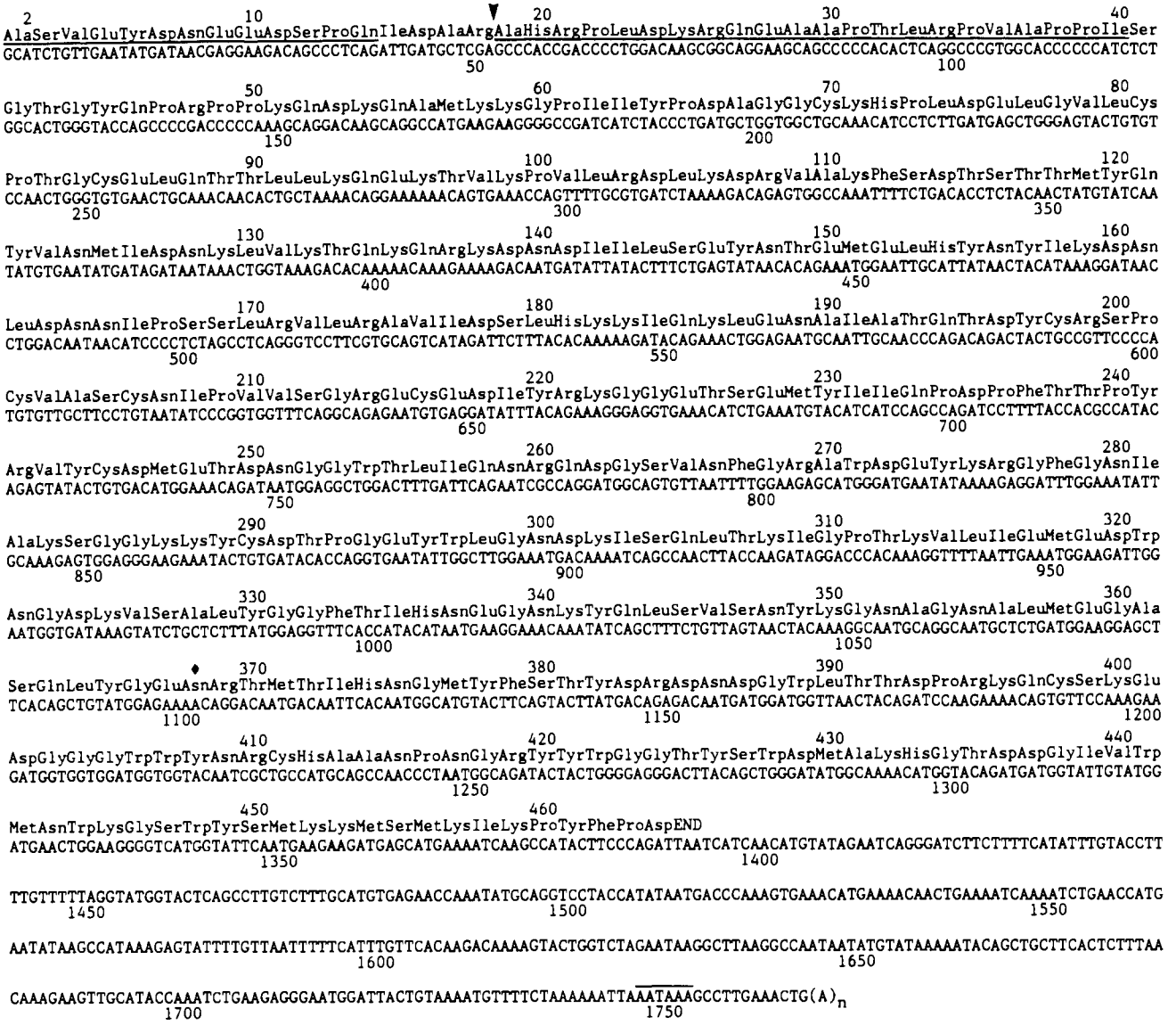

FIGURE 1: cDNA and derived protein sequence for chicken fibrinogen β chain. The cDNA sequence for the chicken fibrinogen β chain, corresponding to almost the entire coding region and the 3′ untranslated region, is presented. The deduced protein sequence begins at amino acid number 2 of the fibrinopeptide B. The arrow denotes the thrombin cleavage site, and the diamond indicates the single glycosylation site. The polyadenylation signal is overlined. Protein sequences that were confirmed directly (i.e., the sequence in fibrinopeptide B and the sequence immediately following the thrombin cleavage site) are underlined.

analogous to those of human fibrin, were dialyzed against 10% acetonitrile–0.1% trifluoroacetic acid, concentrated, and used for automated amino acid sequence analyses with a Model 477A protein sequencer (Applied Biosystems, Foster City, CA).

Fibrinopeptide B Isolation and Amino Acid Sequencing. The clot liquor from the clotting experiment described above was applied to a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA). The cartridge was washed with water, and the fibrinopeptides were eluted with 50% acetonitrile. The eluate was concentrated and applied to FPLC for peptide separation on an RPC PEP 5/5 column (Pharmacia, Piscataway, NJ) equilibrated with 0.2% trifluoroacetic acid. Fibrinopeptides were eluted by use of a gradient of acetonitrile (0–21%), and the fibrinopeptide B peak (eluting at 18.5% acetonitrile) was concentrated. Since Murano et al. (1977) had reported a blocked N terminus of the chicken fibrinogen β chain, the peptide fraction was first treated with pyroglutamate aminopeptidase (sequencing grade; Boehringer Mannheim Biochemicals, Indianapolis, IN) by use of a substrate to enzyme ratio of 10:1 (w/w) according to the manufacturer's instructions. The sample was adjusted to pH 8.0 prior to the addition of enzyme, and digestion took place at 50 °C for 6 h in a buffer consisting of 150 mM sodium phosphate, pH 8.0, 10 mM EDTA, 5 mM dithiothreitol, 2.5% glycerol, and 50 mM guanidine hydrochloride. The sample was applied to a C_{18} cartridge and processed by FPLC as above. The treated peptide eluted at 16–17% acetonitrile, somewhat differently from the untreated peptide, and the collected peak was concentrated and subjected to automated amino acid sequence analysis.

Results and Discussion

The peptide sequence GWWYNR is conserved in all fibrinogen β chains sequenced to date and is located about 50 amino acids from the carboxy terminus (Doolittle, 1983; Chung et al., 1983; Bohonus et al., 1986; Eastman & Gilula, 1989). As an initial probe for isolating a chicken fibrinogen β chain cDNA, an antisense oligonucleotide [C(GT)(AG)-TT(AG)TACCACCA(ACGT)CC] corresponding to this sequence was synthesized. Screening of approximately 5000 plaques of a λgt11 chicken liver cDNA expression library

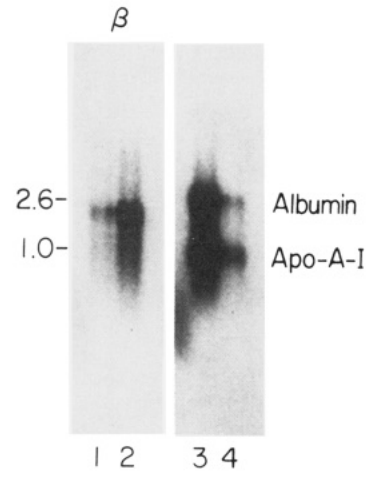

FIGURE 2: Northern analysis of fibrinogen β subunit mRNA in normal and acute-phase chickens. Poly(A)-containing RNA was isolated (Plant et al., 1983) from livers of 3.5-month-old chickens that belonged to either the normal group (lanes 1 and 3) or the group that had been injected 24 h earlier with turpentine to induce the acute-phase response (lanes 2 and 4) as described (Grieninger et al., 1986). RNAs (1 μg) were denatured, electrophoresed on a 1% agarose gel containing 2.1 M formaldehyde, blotted onto nylon filters, and hybridized to radiolabeled cDNA probes. Lanes 1 and 2 were probed with the chicken β fibrinogen 500-bp *Xba*I/*Hin*cII fragment; lanes 3 and 4 were probed simultaneously with chicken albumin and chicken apo-A-I probes (2200 and 750 bp, respectively). The numbers on the left indicate the sizes of the albumin (Gordon et al., 1978) and apo-A-I (Rajavashisth et al., 1987) mRNAs in kilobases.

yielded one positive clone, containing an insert of approximately 900 bp. A 500-bp *Xba*I/*Hin*cII fragment included within the 900 bp was subcloned into the plasmid vector pGEM-3Z and sequenced. A second fibrinogen β chain candidate clone was detected by screening the same cDNA library with a polyclonal antibody to chicken fibrinogen that recognizes all three subunits of the protein (Amrani et al., 1983; Plant & Grieninger, 1986). This second cDNA isolate contained an insert of approximately 1750 bp and was shown, after DNA sequencing, to include the 900-bp cDNA clone. The DNA sequence obtained is shown in Figure 1 along with the deduced protein sequence. It consists of a region that encodes 463 amino acids followed by a 3′ untranslated region of 371 nt, which includes a typical polyadenylation signal 12 nt before the poly(A) sequence. In Northern analysis, the 500-bp *Xba*I/*Hin*cII fragment hybridized to a single mRNA species (of about 2200 nt) that was present in increased amounts during the acute phase response (Figure 2). By contrast, mRNAs for the "negative" acute-phase proteins albumin and apo-A-I showed a substantial decrease. Both observations are consistent with previous studies concerning plasma levels of these proteins (Grieninger et al., 1986).

The beginning of the cDNA sequence corresponds to the codon for alanine at residue 2 of the fibrinogen β chain, a positioning based on comparison with the chicken fibrinopeptide B sequence as determined by direct protein sequencing of purified fibrinopeptide B (see Materials and Methods). Amino acids 2–14 were confirmed in this manner. The first residue in the chicken β chain, known to be blocked (Murano et al., 1977), is either Gln or Glu, since removal of pyroglutamic acid with pyroglutamate aminopeptidase was necessary prior to sequencing. This blocked N terminus is analogous to the initial cyclized glutamine residues found in the duck β chain as well as in the chicken and duck α chains (Takagi et al., 1978; Min et al., 1985).

Figure 3 shows a comparison of the fibrinogen β chain protein sequence from chicken with its counterparts from human (Chung et al., 1983) and lamprey (Bohonus et al., 1986). The N-terminal half contains the the fibrinopeptide and the conserved cysteine motifs bordering a segment of 112 amino acids, with characteristics similar to those of the corresponding α-helical permissive segment in the human β chain. The homologies are particularly striking (81% identity to human and 63% to lamprey) in the C-terminal half, which contains the sole glycosylation site conserved in all fibrinogen β chains (Asn-368 in chicken). Despite the fact that this stretch of ca. 250 amino acids, together with the comparable portion of the γ chain, constitutes the most highly conserved part of the entire fibrinogen molecule, its functional significance is not fully understood. Two functional domains have been assigned to this region thus far: a Ca^{2+} binding site on the γ chain (Varadi & Scheraga, 1986) and a thrombospondin binding domain on the β chain (Bacon-Baguley et al., 1990). Recent recognition of strong homologies to the β and γ chains exhibited over a similarly large C-terminal region by a variety of nonfibrinogen sequences (Weissbach & Grieninger, 1990; Xu & Doolittle, 1990) may pave the way for identification of other functions preserved in this portion of the molecule. Within the broad gene family related through their βγ-homologous C termini, cytotactin (tenascin) is the only nonfibrinogen member currently characterized at the protein level. Like fibrinogen, it is a hexameric secreted protein with subunits held together by interchain disulfide linkages (Jones et al., 1989; Chiquet-Ehrismann, 1990). Functionally, both comprise part of the extracellular matrix and bear the RGD sequence for binding to the integrin family of receptors (Ruoslahti & Pierschbacher, 1987). The physiological importance of their common ancestry may be reflected in the fact that both proteins are associated with wounds during healing (Mackie et al., 1988) and with epithelia of malignant tissue (Dvorak, 1986; Mackie et al., 1987).

An amino acid sequence following the thrombin cleavage site that is conserved in the β chains of human (Chung et al., 1983) and dog (Birken et al., 1975) fibrinogen is identical with chicken β-chain residues 20–25, from which we deduced that the peptide bond hydrolyzed by thrombin lies between Arg-18 and Ala-19 of the chicken sequence. Alignment with duck fibrinopeptide B, which is identical in length with its chicken counterpart (Min et al., 1985), also points to thrombin cleavage at this Arg–Ala bond. However, in all species in which the thrombin cleavage site of the fibrinogen chains has been studied (human, bovine, dog, and lamprey), it was found to contain an Arg–Gly bond; consistent with that pattern, the original published N-terminal sequence of the β subunit of chicken fibrin claims glycine, not alanine, as the first residue of the cleaved chain (Murano et al., 1977).

This matter was clarified by direct sequencing of the β chain of chicken fibrin isolated from clotted plasma fibrinogen (see Materials and Methods). The N-terminal 22 amino acids that were obtained were identical with those deduced from the cDNA, confirming alanine at position 19 and the unusual Arg–Ala thrombin cleavage site.

Thrombin is known to cleave Arg–Ala peptide bonds in several nonfibrinogen proteins, such as apolipoprotein E (Bradley, 1986), actin (Muszbek et al., 1975), and pituitary growth hormone (Graf et al., 1976). This observation, coupled with the fact that amino acid sequences surrounding the actual thrombin cleavage site are important for thrombin specificity (Scheraga, 1983, 1986; Blombäck, 1986; Lord & Fowlkes, 1989), may lessen the impact of the replacement of glycine by alanine in the thrombin cleavage site of the chicken fibrinogen β chain. Nevertheless, chicken fibrinogen is known

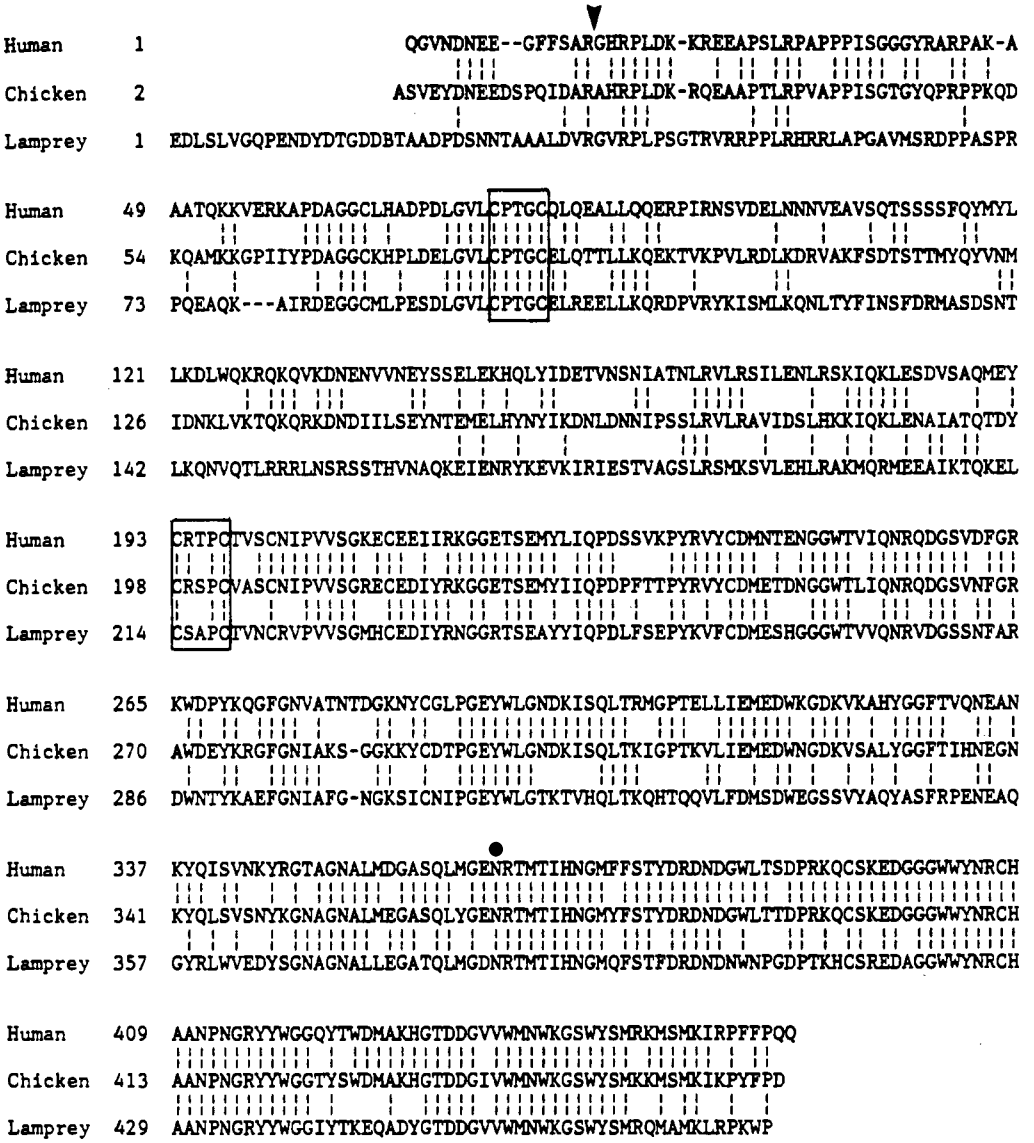

FIGURE 3: Alignment of chicken, human, and lamprey fibrinogen β chain protein sequences. The amino acid sequence for chicken fibrinogen β chain (starting with position 2) is compared to its counterparts in human and lamprey. Single-letter abbreviations for amino acids are used. Identical residues are denoted by dashed vertical lines, and gaps that are introduced to maximize the alignment are represented by dashed horizontal lines. The letter designation B is used to indicate an uncertainty between asparagine or aspartate at position 19 in the lamprey sequence. The arrow denotes the peptide bond cleaved by thrombin in each of the three proteins. The cysteine motifs are boxed, and the diamond indicates the glycosylation site conserved in each of the three β chains. The human sequence is from Chung et al. (1983), and the lamprey sequence is compiled from Bohonus et al. (1986) and Cottrell and Doolittle (1976).

to clot more slowly than mammalian fibrinogen, even when homologous chicken thrombin is used (Chandrasekhar & Laki, 1968). Using human thrombin, Pindyck et al. (1977) showed that release of chicken fibrinopeptide A was comparable to that of its human counterpart but that cleavage of fibrinopeptide B was slower; in addition, formation of chicken α-chain cross-links required a prolonged time interval.

At present, it is not clear whether these phenomena can be traced to those features of the chicken fibrinogen chains found neither in the corresponding genes from species that antedate evolution of the avian lineage (lamprey) nor in ones that evolved more recently (bovine and human). In addition to the unusual Arg–Ala thrombin cleavage site of the β chain reported here, two such features of the chicken α chain may be relevant: an extra cysteine residue situated within one of the conserved cysteine motifs, which could affect the structure of the molecule, and the absence of the oligopeptide repeats that characterize the central region of this chain in other species (Weissbach & Grieninger, 1990). The latter may be particularly significant, since two of the acceptor sites for α-chain cross-linking have been attributed to the oligopeptide repeats in the central portion of the human α chain (Doolittle et al., 1979). Further kinetic studies on chicken fibrinopeptide release and fibrin formation will be required to resolve these issues, all of which may have parallels in clinical cases of abnormal clotting.

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