Coagulation Factor XIIIa Undergoes a Conformational Change Evoked by Glutamine Substrate

STUDIES ON KINETICS OF INHIBITION AND BINDING OF XIIIa BY A CROSS-REACTING ANTIFIBRINOGEN ANTIBODY

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Coagulation factor XIIIa, plasma transglutaminase (endo-γ-glutamine-ε-lysine transferase EC 2.3.2.13) catalyzes isopeptide bond formation between glutamine and lysine residues and rapidly cross-links fibrin clots. A monoclonal antibody (5A2) directed to a fibrinogen α-chain segment 529–539 was previously observed from analysis of end-stage plasma clots to block fibrin α-chain cross-linking. This prompted the study of its effect on nonfibrinogen substrates, with the prospect that 5A2 was inhibiting XIIIa directly. It inhibited XIIIa-catalyzed incorporation of the amine donor substrate dansylcadaverine into the glutamine acceptor dimethylcasein in an uncompetitive manner with respect to dimethylcasein utilization and competitively with respect to dansylcadaverine. Uncompetitive inhibition was also observed with the synthetic glutamine substrate, LGPGQSKVIG. Theoretically, uncompetitive inhibition arises from preferential interaction of the inhibitor with the enzyme-substrate complex but is also found to inhibit γ-chain cross-linking. The conjunction of the uncompetitive and competitive modes of inhibition indicates in theory that this bireactant system involves an ordered reaction in which docking of the glutamine substrate precedes the amine exchange. The presence of substrate enhanced binding of 5A2 to XIIIa, an interaction deemed to occur through a C-terminal segment of the XIIIa A-chain (643–658, GSDMTVTVQFT-NPLKE), 55% of which comprises sequences occurring in the fibrinogen epitope Aα-(529–540) (GSESGLFTNTEKE). Removal of the C-terminal domain from XIIIa abolishes the inhibitory effect of 5A2 on activity. Crystallographic studies on recombinant XIIIa place the segment 643–658 in the region of the groove through which glutamine substrates access the active site and have predicted that for catalysis, a conformational change may accompany glutamine-substrate binding. The uncompetitive inhibition and the substrate-dependent binding of 5A2 provide evidence for the conformational change.

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1 The abbreviations used are: fXIII, factor XIII; XIIIa, the enzymically active form of blood coagulation fXIII; XIIIa, A-chains of fXIII; rXIIIa, recombinant XIIIa A-chains; 5A2, anti-fibrinogen(ogen) α 529–539; mAb, monoclonal antibody; FPA, fibrinopeptide A (fibrinogen Aα 1–16); TBS, Tris-buffered saline with EDTA; TBSTw, Tris-buffered saline with Tween.
**Antifibrinogen Δα-(529–539) mAb/Factor XIIIα**

**MATERIALS AND METHODS**

Proteins and Reagents—Initial studies on inhibitory effects of 5A2 were carried out directly in plasma. Kinetic studies used ZymoGenetics Inc. (Seattle, WA) (rXIIIα) (19). Synthetic substrates for XIIIa were used as supplied in Berichrom® XIIIa assay kits except when supplemented with the glutamate substrate LPGGQS/KVIG from Behring Werke (Marburg, Germany). Bovine N,N-dimethylated casein, dansylphenylalanine-converting enzyme mAb B932 (20) was generously provided by Dr. S. M. Danilov (Russian Cardiology Research Center, Moscow). Rabbit anti-XIIIa antibodies were generated using rXIIIa as antigen and purified by elution (0.35 mM citrate, pH 3.5) from protein A-Sepharose (Amersham Pharmacia Biotech). Except where indicated, all protein solutions were in Tris-buffered saline containing 0.1 mM EDTA (TBSE) at pH 7.4.

Human fibrinogen was purified from the donor plasma by sequential precipitations with ethanol, glycine, and ammonium sulfate to a capric acid as described (24). Where indicated, it was immunochromically freed of XIIIa by passing it (36 mg, 3 ml) through a 10-ml column of affinity-purified rabbit anti-XIIIa antibody (20 mg) that was linked to beaded 2% glyoxylo-agaroose gel (Global Imports, Tampa, FL) essentially as described (25). The columns were reused after a flush with 5 ml of 4 M guanidine-HCl, pH 9.0 containing dithiothreitol (2.5 mM) and the concentrations of preactivated XIIIa in precipitations with ethanol, glycine, and ammonium sulfate/TBSE at pH 7.4.

Effect of 5A2 on Cross-linking of Fibrinogen—These studies simply involved adding calcium (5 mM) and dithiothreitol (2.5 mM) in the presence and absence of added 5A2 (4 mg/ml) to the fibrinogen (2 mg/ml) prior to the preparation before removing the contaminating XIIIa from the fibrinogen. The observed cross-linking was verified as due to the presence of active XIIIa in the fibrinogen preparation, because it occurred without thrombin activation and in the presence of hirudin, it did not change during a parallel incubation (6 h, 22°C) with 8 mg/ml 5A2 added, and it did not occur at all after passing the fibrinogen through the anti-XIII affinity gel.

Cross-linked products were separated by SDS-polyacrylamide gel electrophoresis of thiold-reduced specimens and identified after Coomassie staining by comparison with previous electropherograms identifying the polypeptide chain compositions of the products (26).

**Activation of rXIIIa**—To preactivate the rXIIIa (2 mg/ml), it was incubated (15 min, 37°C) with thrombin (0.5 units/ml) at pH 7.4 in the presence of CaCl₂ (2 mM) and a trace amount of fibrinogen (2 mg/ml). The reaction was then terminated by adding calcium (5 mM) and dithiothreitol (2.5 mM) calcium for 30 min at 37°C, and the thrombin was subsequently inactivated with 0.3 units of hirudin before washing and blocking for enzyme-linked immunosorbent assay measurements.

**Assays of XIIIa Activities Using Dimethylcasein As the Glutamine Substrate and Dansylcadaverine As the Amine Substrate**—This assay uses a synthetic decapeptide (LPGGQS/KVIG) as glutamine substrate and glycine ethyl ester as the amine substrate and measures transglutaminase activity from the rate of ammonia release from the peptide using an auxiliary enzyme system (28). The ammonia release is followed colorimetrically from consumption of NADH as the ammonia is driven into reaction with α-ketoglutarate by glutamate dehydrogenase, all of these components apart from NADH being packaged in the "reporting" solution of the Berichrom® XIIIa kit. The assays were carried out according to the recommended procedure of the supplier except for two modifications made to characterize the kinetics of 5A2 inhibition in relation to LPGGQS/KVIG concentrations. We substituted rXIIIa/5A2 mixtures for the plasma sample for measuring plasma XIIIa in the kit. Secondly, since the reporting solution contained a saturating level of LPGGQS/KVIG, we diluted it in half and varied the concentration of LPGGQS/KVIG by supplementing the diluted solution with added peptide. As determined with the peptide reconstituted to its original level, apparent reaction velocities, both in the presence and absence of 5A2 reported with the diluted solutions, were consistently one-half those reported with the undiluted solution due to the lower concentration of reporting enzyme (glutamate dehydrogenase) in the diluted system.

**Preparation of C-terminal-truncated 51-kDa Fragment of rXIIIa**—This tryptic derivative of rXIIIa was prepared essentially as described by Greenberg et al. (29). Briefly, the digest consisted rXIIIa (2 mg/ml), trypsin (1 mg/ml), and CaCl₂ (10 mM) at 37°C. SDS-polyacrylamide gel electrophoresis showed that full conversion of rXIIIa to the 51-kDa fragment required 2.5 h of incubation under these conditions. The digestion was terminated by adding Aprotinin® (0.14 mg/ml). The electropherograms showed that none of the 80-kDa parent rXIIIa remained unconverted. A shorter incubation of 1.5 h left 75% converted. The 51-kDa fragment precipitated out of solution during the incubation, was centrifuged, and resuspended in TBSE, and the suspension was divided into 0.1-mI aliquots, which were stored frozen. Just before assay, the suspension was thawed, and admixed with an equal volume of 6 μl urea (3 M final) to dissolve the protein immediately before measuring its activity.

**Enzyme-linked Immunosorbent Assay for Binding**—Nunc immune plates (Vanguard Intl., Neptune, NJ) were coated with rXIIIa in TBBS (10 μg/ml, 100 μl/well, 18 h, 4°C), washed with TBS, and blocked with 3% bovine serum albumin in TBS/Tween (TBSTw). Primary antibodies, either 5A2 or control anti-FPA mAbs (100 μg/ml, 100 μl/well), were added in 0.1% bovine serum albumin in TBSTw supplemented with either Behrichrom substrate or blank (10 μl/well). After 18 h of equilibration at 4°C followed by a TBSTw wash, the retained 1° antibody was fixed in place with cold 0.25% glutaraldehyde in phosphate-buffered saline (100 μl/well, 4°C, 10 min), and the plates were washed (TBSTw) and quenched 0.1% bovine serum albumin in TBSTw (200 μl/well, 37°C, 10 min). After further washing, secondary reporter antibody (horseradish peroxidase-goat-anti-mouse Ig at 1:1000 dilution, 100 μl/well) was added, and the excess was washed away after 2 h at 23°C. Retained 2° antibody was assessed from peroxidase activity toward orthophenylenediamine.

**RESULTS**

Inhibitory Effects of mAb 5A2 on Fibrinogen α- and γ-Chain Cross-linking—In the course of studies on the effect of 5A2 on the cross-linking of fibrinogen we observed (Fig. 1) that γγ-dimer formation was inhibited to a degree comparable to the subsequent (26) incorporation of fibrinogen α-chains into hybrid αγγ-trimers. The inhibition of γ-chain cross-linking was not detected in our preceding study, which characterized only the effect of 5A2 on end-stage plasma clots (20). The inhibition of γ-chain cross-linking together with searches indicating that there was no homology between the 5A2 epitope on fibrinogen (20) and the amino acid sequences in the fibrinogen γ-chains, prompted us to investigate whether the inhibitory effect of 5A2 was arising from a direct interaction with XIIIa. We then proceeded to examine the effects of 5A2 on substrates other than fibrinogen.

**Kinetics of Dansylcadaverine Incorporation into Dimethylca-**

—This reaction was inhibited by 5A2 in a concentration-de-
The first lane (at left) shows the noncross-linked α, β, and γ chains in the parent fibrinogen stored in 0.1 mM EDTA. Cross-linking initiated by adding calcium and dibiotin in the absence of 5A2 (center lane) caused a nearly complete incorporation of γ-chains into characteristic γγ-dimers and subsequent mixed αγγ oligomers with accompanying substantial disappearance of noncross-linked α-chains. In the presence of 5A2 (right lane), the cross-linking of both γ- and α-chains was appreciably arrested, as evidenced by the diminished disappearance of noncross-linked chains and reductions in the levels of both γγ-dimers and αγγ-trimers, the percentage change in γ- and α-chains products being of comparable magnitudes. The restoration of noncross-linked γ-chains appears greater than would be expected from the reduced levels of cross-linked products because 5A2 heavy chains co-migrated in that band. The light chains from added 5A2 appear at the bottom of the lane.

The Lineweaver-Burk plots of 1/v versus 1/[dimethylcasein] at 5A2 concentrations ranging to 0.6 mg/ml (Fig. 2). A replott of the 1/v intercepts versus the 5A2 concentrations was linear, with a negative intercept at 5A2 concentrations corresponding to a $K_i$ of 0.8 mM (Fig. 2, inset). The parallel Lineweaver-Burk plots and linear replots conformed with a pattern of inhibition termed “uncompetitive,” where the inhibitor is directed principally toward the enzyme-substrate complex (30), the glutamine substrate in this instance. These effects were specifically caused by 5A2 because no inhibition was observed with 7F8 and were also specifically directed to XIIIa because no inhibitory effects of 5A2 were observed toward transamination of these substrates by tissue transglutaminase (negative results not shown).

The Lineweaver-Burk plots of 1/v versus 1/[dansylcadaverine], on the other hand, yielded slopes that varied with the 5A2 concentrations (Fig. 3) and conformed with a competitive mode of inhibition. A linear replot of the slopes versus 5A2 concentrations intercepted the negative 5A2 axis at $K_i = 0.4 \mu M$ (Fig. 3, inset).

Transamination of Berichrom® Decapeptide LGPGQSKVIG with Glycine Ethyl Ester—As illustrated (Fig. 4), 5A2 exhibited a concentration-dependent inhibition of XIIIa-catalyzed release of ammonia from the decapptide in a pattern consistent with the uncompetitive mode of inhibition observed with the other nonfibrinogen-related glutamine substrate, dimethylcasein. The 1/v intercepts (corresponding to values for 1/$V_{max}$) at 1/S = 0 on the Lineweaver-Burk plots varied linearly with 5A2 concentrations (Fig. 4, inset). Extrapolating this replot to the base-line 1/$V_{max}$ at 0 yielded an estimate of 0.9 $\mu M$ for the value of $K_i$, which could be off by a factor of two (95% confidence interval) because of the length of the extrapolation.

Homology Searches—The inhibitory effects of 5A2 on utilization of nonfibrinogen substrates and the absence of effect on tissue transglutaminase suggested that 5A2 was interacting with a segment of XIIIa with a sequence similar to that of the fibrinogen epitope. The fibrinogen epitope was identified from CNBr and tryptic fragments to be located in the region of $\alpha\alpha\alpha$ (529–539) mAb/Factor XIIIa.

Fig. 1. Electropherograms illustrating the inhibitory effect of 5A2 on both γ- and α-chain cross-linking of fibrinogen by XIIIa. The first lane (at left) shows the noncross-linked α, β, and γ chains in the parent fibrinogen stored in 0.1 mM EDTA. Cross-linking initiated by adding calcium and dibiotin in the absence of 5A2 (center lane) caused a nearly complete incorporation of γ-chains into characteristic γγ-dimers and subsequent mixed αγγ oligomers with accompanying substantial disappearance of noncross-linked α-chains. In the presence of 5A2 (right lane), the cross-linking of both γ- and α-chains was appreciably arrested, as evidenced by the diminished disappearance of noncross-linked chains and reductions in the levels of both γγ-dimers and αγγ-trimers, the percentage change in γ- and α-chains products being of comparable magnitudes. The restoration of noncross-linked γ-chains appears greater than would be expected from the reduced levels of cross-linked products because 5A2 heavy chains co-migrated in that band. The light chains from added 5A2 appear at the bottom of the lane.

Fig. 2. The Lineweaver-Burk plots characterizing 5A2 as an uncompetitive type of inhibitor of the utilization of glutamine substrate (dimethylcasein) by XIIIa. Reaction velocities (v) were determined from initial rates of increase in fluorescence arising (27) from incorporation of the dansylcadaverine at fixed concentration into dimethylcasein at varying concentrations in the presence and absence of 5A2. The presence of 5A2 diminished reaction velocities (indicated by the upward shifts in the intercepts on the 1/v axis where $1/v = 1/V_{max} i$) but did not alter the slopes, a pattern typical of uncompetitive inhibition. The inset shows the linear relationship between the 1/v-axis intercepts versus the 5A2 concentrations, which extrapolate to $-0.8 \mu M$ for the value of $K_i$.

Fig. 3. The Lineweaver-Burk plots characterizing 5A2 as a competitive type of inhibitor of the utilization of amine substrate (dansylcadaverine) by rXIIIa. Reaction velocities (v) were determined as for Fig. 3, but the concentration of dimethylcasein was fixed at a high level, and concentrations of dansylcadaverine were varied in the presence and absence of 5A2. Here, the presence of 5A2 shifted the slopes rather than the 1/v axis intercepts, a pattern typical of competitive inhibition. The inset shows the linear relationship between the slopes versus the 5A2 concentrations, which extrapolate to $-0.4 \mu M$ for the value of $K_i$. 

CNBr and tryptic fragments to be located in the region of $\alpha\alpha\alpha$ (529–539), the only fragment from the combined digests that substantially inhibited (18%) binding of 5A2 to fibrinogen $\alpha$-chains (20). Searches for similarities between the sequence of this peptide and the amino acid sequences (16, 31) in XIIIa were made using both the PROPHET (Bolt Beranek and Newman, Inc., Cambridge MA) and the BLAST network service (NCBI). Both searches found a segment near the C-terminus of
the Aα-chain 643–658 of XIIIa that consists in part (55%) of sequences occurring in the region of the fibrinogen Aα-chain epitope, and no other regions were found (3% homology) in either XIIIa, fibrinogen, or casein. XIIIa:643GS—DTMVTQVFT—NPLKE; Fgn Aα: 529GS—GDMTVTVQFT—NPLKE; Fgn Aα: 529GS—GDMTVTVQFT—NPLKE; Fgn Aα: 529GS—GDMTVTVQFT—NPLKE; Fgn Aα: 529GS—GDMTVTVQFT—NPLKE; Fgn Aα: 529GS—GDMTVTVQFT—NPLKE.

No Effect on an Enzymically Truncated XIIIa—To test the importance of the C-terminal domain for the inhibitory effects of 5A2, we examined its effect on cross-linking of a tryptic fragment of 5A2 lacking the putative cross-reacting domain. Greenberg et al. (29) observed that a tryptic fragment of XIIIa comprising residues Gly 38-Lys513 retained 20% of its initial activity. On trypsinizing rXIIIa we obtained a pure 51-kDa fragment that precipitated during the digestion. The fragment exhibited transglutaminase activity in the Berichrom® assay when it was admixed (0.015 mg/ml) with the substrates immediately after dissolving it in 3 M urea. Unlike intact XIIIa, activity of the fragment was unaffected by 5A2 (Fig. 5). This result supported the hypothesis that 5A2 was inhibiting intact XIIIa by interacting with the segment in the XIIIa C-terminal domain, residues 643–658 with sequence similar to the 5A2 epitope.

Binding of 5A2 with rXIIIa—Attempts to detect binding of 5A2 to plated rXIIIa by enzyme-linked immunosorbent assay methods failed until we resorted to fixing the initially bound 5A2 with glutaraldehyde to prevent dissociative losses during the assay when it was admixed (0.015 mg/ml) with the substrates immediately after dissolving it in 3 M urea. Unlike intact XIIIa, activity of the fragment was unaffected by 5A2 (Fig. 5). This result supported the hypothesis that 5A2 was inhibiting intact XIIIa by interacting with the segment in the XIIIa C-terminal domain, residues 643–658 with sequence similar to the 5A2 epitope.

Binding of 5A2 with rXIIIa—Attempts to detect binding of 5A2 to plated rXIIIa by enzyme-linked immunosorbent assay methods failed until we resorted to fixing the initially bound 5A2 with glutaraldehyde to prevent dissociative losses during
exposure (30 min) to secondary, reporter antibody. This approach detected binding of 5A2 to the rXIIIA zymogen substantially above that of a control mAb, anti-FPA (Fig. 6). Activating the rXIIIA with thrombin promoted binding of both the 5A2 and anti-FPA mAbs (5A2 more so), whether the activation was performed before plating the rXIIIA (columns labeled XIIIa) or after plating (columns labeled XIIIA*). We suspect that the enhanced binding of the anti-FPA was due to an effect of thrombin, because plating thrombin caused some retention of the anti-FPA not observed with 5A2. Unlike the binding of anti-FPA, the 5A2 binding became substantially enhanced further when the synthetic substrate from the Berichrom® XIIIa kit was added to the medium during incubation. The added substrate also caused a slight increase in binding to the zymogen, which could conceivably be due to the normal development of low levels of activity by zymogen without thrombin treatment. Essentially the same enhancing effects of added substrate were reproducibly obtained in repeated experiments. The enhanced binding of 5A2 after activation with thrombin and the further enhancement after adding substrate were viewed as indications that substrate binding was inducing a conformational change leading to greater exposure of the epitope for 5A2 binding.

DISCUSSION

The C-terminal domain of XIIIA has been shown by Greenberg et al. (29) to have some influence on the transglutaminase activity of the enzyme, as judged by the substantial (80%) loss of activity after removing the domain with trypsin. Attempts to probe its function immunochemically have proven difficult, because it is poorly immunogenic (29). As described here, we serendipitously found that an antibody (5A2) directed to a C-terminal domain of the fibrinogen Aα-chain (529–539) inhibits XIIIs in a manner dependent on the C-terminal domain of the enzyme. This inhibition is deemed to involve a cross-reactivity with residues 643–658 in the XIIIA chain, which share homology with the fibrinogen epitope, and further appears to be partially dependent on interaction with XIIIs complexed with substrate. The modes of inhibition have important implications for the structure and activity of XIIIs.

The crystallographic structure of XIIIA was recently elucidated (17, 32, 33). As illustrated (Fig. 7), the cross-reacting segment 643–658 forms a strand in the outer β sheet of the C terminus of the barrel 2 domain of XIIIA. The lysine 657 residue is located at the gap between the barrel 1 and barrel 2 domains, which has been identified as one of two possible loci through which glutamine substrates gain access to the catalytic site of the enzyme (32). The inhibition of utilization of glutamine substrates (both the dimethylcasein and the synthetic peptide LQPQQSKVIG) was uncompetitive, an indication (30) that the principal mode of that inhibition was directed to the enzyme-glutamine complex. Any other mode of enzyme-inhibitor interaction is incidental. In essence, the glutamine binding acts as an activator of the catalytic conformational change that enables utilization of the amine substrate. Utilization of the amine substrate was inhibited competitively, probably not because 5A2 acts as an analog of the amine substrate, but, more likely, because the amine substrate cannot bind to the ternary glutamine donor-XIIIs-5A2 complex. The independent observation of increased 5A2 binding to XIIIs in the presence of glutamine substrate is consistent with that explanation. Thus, these two modes of inhibition indicate that the transamination reaction is an ordered event dependent on docking of the glutamine substrate to trigger reaction with subsequent binding of the amine substrate.

Binding of 5A2 to XIIIs was enhanced by co-incubation with the Berichrom® XIIIa substrate system, an indication that substrate binding enhances exposure of the 5A2 epitope on XIIIs. It had been predicted that a conformational change accompanying the glutamine binding functions in the catalytic activity of the enzyme (32). Crystal structures of rXIIIA both before and after activation with either thrombin or high concentrations of calcium (34, 35) led to the inference that the conformational change that must accompany catalysis arises from the docking of the glutamine substrate. Our findings provide evidence for a substrate-induced conformational change and primacy of glutamine binding in the catalytic reaction.

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