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Peptide-Derivatized Albumins That Inhibit Fibrin Polymerization

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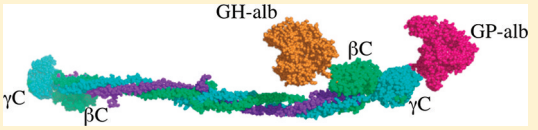
Supporting Information

ABSTRACT: Synthetic peptides patterned on sequences that appear during thrombin proteolysis of fibrinogen are known to influence fibrin formation in very different ways. A-Knob sequences (GPR-) inhibit polymerization, but B-knob sequences (GHR-) can actually enhance the process. We now report that when such peptides are attached to albumin carriers, both knob conjugates inhibit fibrin formation. In contrast, the 2-aminoethylthiol−albumin conjugate control enhances the polymerization to the same degree as albumin. The peptide AHRPam, which is known to bind exclusively to the βC holes of fibrinogen/fibrin, nullifies the inhibitory effects of the GHRPYGGGCam−albumin conjugate on fibrin polymerization, indicating that the inhibition was exclusively due to interactions with βC holes. AHRPam was much less effective in countering inhibition by the GPRPGGGGCam−albumin conjugate, suggesting that the observed effects with this conjugate involve mainly the γC holes of fibrin/fibrinogen. This study demonstrates that peptides modeled on fibrin polymerization knobs tethered to albumin retain their capacity to interact with fibrinogen/fibrin and may prove useful as inhibitors of clotting in vivo.



Fibrinogen is changed into fibrin by the thrombin-catalyzed release of fibrinopeptides.[1] During the process, two new pairs of amino termini are exposed in the central portion of the molecule.[2] In mammals, one pair of the new terminal sequences begins with GPR ("A-knobs"); the other pair begins with the sequence GHR ("B-knobs"). The A-knobs bind to "holes" in the γC domains of neighboring fibrinogen molecules and B-knobs to holes in βC domains.[3−5] These knob−hole interactions result in noncovalent polymers in which the central region of one fibrin molecule is associated with the ends of two neighboring molecules.[2,5] The rate of polymerization is affected by numerous factors, including pH, ionic strength, calcium ions, temperature, thrombin concentration, etc.[6]

Small peptides with the beginning sequence GPR, corresponding to the A-knob, inhibit fibrin polymerization, but those beginning with the B-knob sequence GHR do not; in fact, B-knobs tend to enhance the turbidity of gels.[3,4]

Synthetic A-knobs have been widely used in vitro to prevent fibrin gel formation. They have not proven to be useful in vivo, however, because they are rapidly cleared from the circulation and have low association constants.[7] Attaching the peptides to a macromolecule carrier offers the possibility of extending their persistence in the circulation. One obvious choice of carrier is albumin, a protein present in mammalian blood plasma at high concentrations that has been widely used for therapeutic purposes.[8] To be effective in preventing the formation of clots, derivatives need to be monovalent. Because fibrinogen is a dimer, carriers with more than one peptide knob per molecule induce precipitation. Albumin's single free sulfhydryl provides a means of preparing monosubstituted peptide−albumin conjugates.

To this end, three different albumin conjugates were made and tested: GPRPGGGGCam−albumin (GP−alb), GHRPYGGGCam−albumin (GH−alb), and, third, a simple cysteamine derivative (ae−alb), the purpose of which was to block the albumin free sulfhydryl and serve as a control along with underivatized albumin. The peptides were designed with polyglycine tethers that allow the knobs to extend away from the carrier when the carboxy-terminal cysteine sulfhydryls are used as the means of attachment to albumin. Additionally, the amino-terminal sequences GPRP- and GHRPY-, which differ slightly from the natural human A- and B-knob sequences, respectively, were used because they were previously found to bind more strongly to fibrinogen. The A-knob peptide had proline at position 4 instead of the native valine,[3] and the B-knob peptide had tyrosine at position 5 instead of leucine.[9]

Both peptide knob−albumin conjugates were found to inhibit fibrin polymerization at concentrations well below the concentration of albumin in plasma (500−600 μM). In contrast, the controls, similar concentrations of albumin and its conjugate with cysteamine, enhanced fibrin polymerization, as has been reported for albumin in the past.[10−13]

MATERIALS AND METHODS

The peptide amides GPRPGGGGCam and GHRPYGGGCam were purchased from GenScript and Sigma-Genosys, respectively. The carboxyamides were chosen to prevent the negative charge of the carboxyl group. As has been previously reported,[14] the albumin thiolate anion reacts slowly with negatively charged disulfides. Cystamine dihydrochloride was obtained from Aldrich. Bovine serum albumin, fraction V, crystalline, was purchased from Cal Biochem and used without further purification. Human thrombin was purchased from

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Enzyme Research Corp. Fibrinogen was prepared from outdated human blood plasma as described previously [15]

Sulfhydryl Determination. Sulfhydryls were assessed by a variation of the Ellman method, cystamine being employed as a "mediator".[16] Solutions of 10 mM Ellman's reagent and 10 mM cystamine, both in 0.1 M sodium phosphate buffer (pH 7.0), were prepared separately. The Ellman's solution was added first to the samples to be analyzed, followed by the addition of the cystamine solution. Absorbance readings were taken at λ = 412 nm.

Peptide–Albumin Conjugates. In a typical preparation of GP–alb or GH–alb, 120 μL of 26 mM peptide (monomer equivalent) in water was added to 2.0 mL of 0.72 mM albumin (70% free sulfhydryl) in 2 M NaCl and 0.1 M sodium phosphate buffer (pH 7.2) and the mixture incubated at 37 °C for 45 h. The peptide to albumin-thiol ratio was 3:1. In some preliminary experiments, the ratio was only 1.5:1, under which conditions the reaction time was extended to 68 h. The extent of reaction was followed by the decrease in the level of free sulfhydryl in the reaction mixture (FIgure S1 of the Supporting Information). Any peptide thiol initially present or produced during the course of the reaction oxidized quickly to its disulfide.[17,18]

Upon completion, reaction mixtures were dialyzed against large volumes of 0.15 M NaCl and 0.05 M imidazole buffer (pH 7.0) at room temperature for 2 h followed by dialysis for 5 days at 4 °C with one or more changes of buffer per day to ensure full removal of excess peptide remaining at the end of the reaction.

Similar procedures were used to prepare the "treated albumin" and the 2-aminoethylthiol–albumin conjugate controls, i.e., the reaction procedure with no added peptide and substitution of cystamine for peptide. Because the reaction of albumin with cystamine is considerably faster than with the more hindered peptides, the preparation of the 2-aminoethyl–albumin mixed disulfide can be completed at room temperature in a considerably shorter reaction time. Nevertheless, the procedure described above was used to ensure that it was produced under the same conditions that were used to prepare the peptide–albumin conjugates.

Protein concentrations were determined by the Lowry method.[19] In all cases, protein recovery was greater than 90%. In most instances, the reaction course of peptides with albumin was complete and no detectable free sulfhydryl remained, but in a few preparations, small amounts of free sulfhydryl were detected. The treated albumin control, which went through the same process but without the provision of a ligand, ended up with 60–65% free sulfhydryl compared with 70% for the starting albumin. In all cases, peptide–albumin conjugate concentrations are given as total protein as determined by the Lowry method, even though only 60–70% of the molecules have covalently bound peptide.

Fibrin Polymerization Assays. Thrombin–fibrinogen clotting assays were conducted at room temperature on 96-well microtiter plates and monitored with a TECAN M200 automatic plate reader at λ = 450 nm. All reaction components were dissolved separately in 0.15 M NaCl and 0.05 M imidazole buffer (pH 7.0) and then combined on the microtiter plate, with thrombin added last by means of an eight-channel pipetteman to initiate the reaction. The reaction course tended to vary somewhat from day to day, depending on the particular batch of fibrinogen or thrombin (Figure S2 of the Supporting Information). All figures represent averages of duplicate runs obtained in single experiments with the same thrombin and fibrinogen samples. The reference reaction in all figures is the thrombin-catalyzed fibrin polymerization without added protein or peptide. The linear nature of the concentration dependence was shown by comparing turbidity profiles with the reference curve at every time point to give "turbidity ratios". These were >1.0 for control albumin preparations and <1.0 for peptide-derivatized albumins.

Modeling. Hypothetical models of the peptide–albumin conjugates interacting with fibrinogen were made with the O modeling program package.[20] In each case, the appropriate peptide in an extended form was positioned with its carboxy-terminal cysteine adjacent to Cys34 of albumin [Protein Data Bank (PDB) entry 2VUE]. The amino-terminal GPRP sequence of the A-knob conjugate was superposed on the crystallographically determined synthetic peptide in the γC hole of fibrinogen (PDB entry 3GHG); the same procedure was used with the GHRP region of the B-knob conjugate and the βC hole of fibrinogen. Illustrations of the final models were made with PyMol.[21]

RESULTS

Inhibition of Fibrin Polymerization by Knob–Albumin Conjugates. Consistent with numerous past reports,[10−13] untreated albumin enhances fibrin polymerization (Figure 1A). The effect was concentration-dependent over the range of 50–100 μM (Figure 1A, inset). Treated albumin and the ae–alb conjugate controls enhance polymerization to the same degree as untreated albumin (Figure 1B).

In contrast, both knob–albumin conjugates, GH–alb and GP–alb, inhibit fibrin polymerization (Figure 2A). For both conjugates, the inhibition shows a concentration dependence, with significant effects at protein concentrations as low as 100 μM (Figure 2B).

Identifying the Binding Sites. Ascertaining the holes on fibrinogen to which the knob–albumin conjugates were binding was worthwhile. Because the free peptide AHRPam is known to bind only to βC holes of human fibrinogen,[22] it was used as a competitive inhibitor to investigate the site of binding for the two conjugates. In this regard, a 20-fold molar excess of AHRPam completely nullifies the inhibitory effect of GH-alb on fibrin polymerization (Figure 3A). In contrast, the same 20-fold excess of free peptide only slightly reduces the inhibition caused by GP–alb (Figure 3B). The results indicate that the GH–alb inhibition effect is completely due to binding to the βC hole, while that caused by GP–alb is due mainly to binding to the γC hole.

Effectiveness of Free and Tethered A-Knobs. Tethering the A-knob to albumin does not impair its effectiveness for inhibiting fibrin formation. At comparable molar concentrations, suitably corrected for the initial free sulfhydryl content of the albumin, the degrees of interference with polymerization by GP–alb and the free peptide GPRPam are approximately the same, allowance being made for GP–alb containing 60–70% peptide (Figure 4).

DISCUSSION

Both peptide knob–albumin conjugates GP–alb and GH–alb inhibit fibrin polymerization. In contrast, the two albumin controls, treated albumin and the ae–alb conjugate, both enhanced polymerization to the same degree as "untreated albumin" as has been previously reported and attributed to

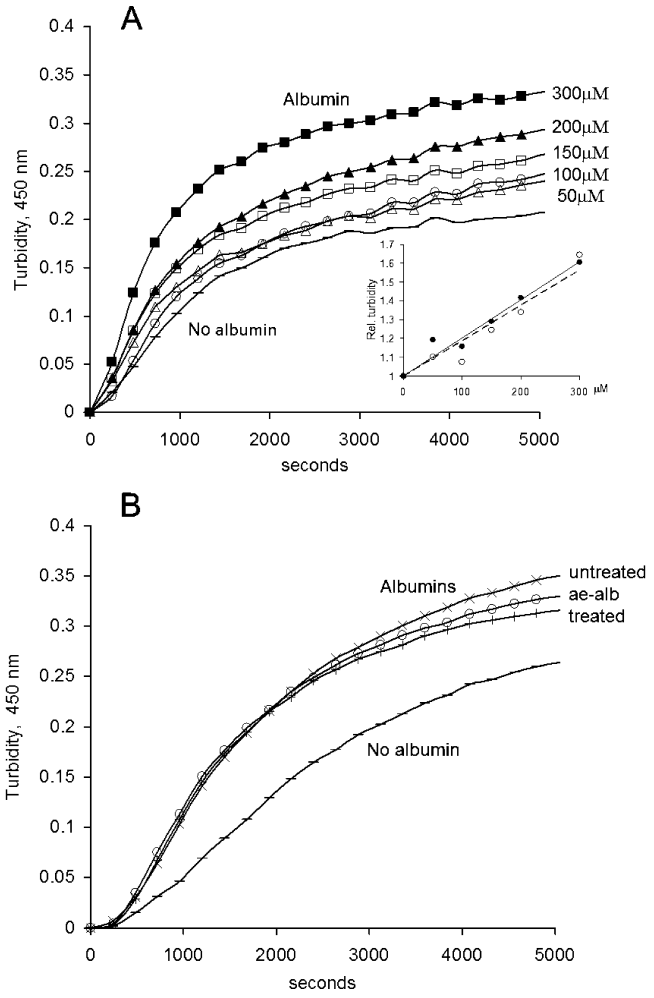

Figure 1. (A) Concentration dependence of the effect of bovine albumin on fibrin polymerization. Final albumin concentrations were 300 (■), 200 (▲), 150 (□), 100 (△), 50 (●), and 0 μM ("reference") (—). The inset shows turbidity ratios vs the concentration of albumin at 2400 (○) and 5000 s (●). (B) Comparison of untreated albumin with 2-aminoethylthio–albumin (ae–alb) and treated albumin (see the text). Final concentrations were as follows: 220 μM ae–alb (○), 260 μM treated albumin (+), 220 μM untreated albumin (×), and no added albumin (reference) (—). Reactions were conducted in 0.15 M NaCl and 0.05 M imidazole buffer (pH 7.0) with 1 mg/mL fibrinogen and 4.3 nM thrombin. Turbidity was measured at λ = 450 nm.

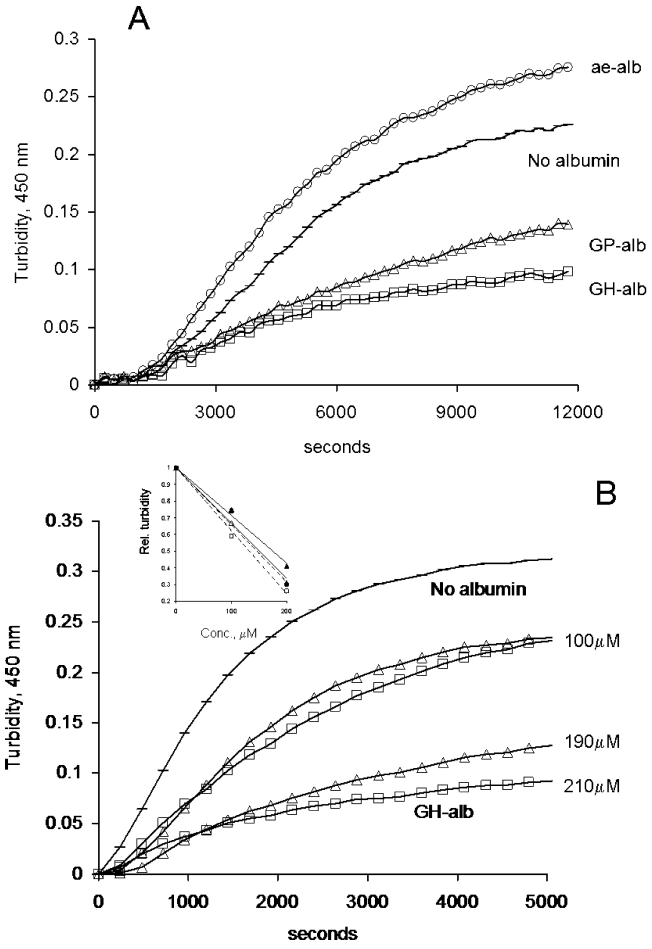

Figure 2. (A) Knob–albumin conjugates inhibit the formation of fibrin under conditions where the ae–alb control enhances its formation. Final concentrations were as follows: 200 μM GH–alb (□), 200 μM GP–alb (△), 200 μM ae–alb (○), and no added albumin (reference) (—). The thrombin concentration was 1.7 nM and the fibrinogen concentration 1 mg/mL. (B) Concentration dependence of the effect of knob–albumin conjugates on the formation of fibrin: 100 and 210 μM GH–alb (□), 100 and 190 μM GP–alb (△), and no added albumin (reference) (—). The inset shows turbidity ratios at two concentrations of each conjugate at 2400 s [GP–alb (△) and GH–alb (□)] and 5000 s [GP–alb (▲) and GH–alb (■)].

solvent exclusion effects.[9−12] The fact that the knob–albumin conjugates give the exact opposite response underscores their effectiveness as polymerization inhibitors.

Although small peptides beginning with the sequence GPR, corresponding to the A-knob of fibrin molecules, are well-known to retard the polymerization of fibrin by associating with γC holes on fibrin/fibrinogen, peptides corresponding to the B-knob sequence GHR actually increase the turbidity of fibrin when associating with the βC holes on fibrin/fibrinogen.[3,4] Consequently, it is noteworthy that both GP–alb and GH–alb inhibit polymerization to comparable degrees, in contrast to the differing effects of the corresponding free peptides. Clearly, the steric bulk of the albumin moiety is preventing the close approach of other fibrin/fibrinogen molecules needed for polymer formation. Models based on the crystal structures of fibrinogen and albumin show that knob–albumin conjugates associated with either hole would block the close approach of additional fibrin/fibrinogen molecules and hinder polymer formation (Figure 5). In this respect, it is informative that GP–alb and GPRPam inhibit to similar degrees at comparable concentrations (Figure 3B). This comparability suggests that in both conjugates the amino acid tethers are sufficiently flexible and can be sufficiently extended from the albumin moiety to allow the knobs to associate with the fibrinogen holes to a degree similar to that of the free synthetic knob peptides.

The competition experiments with the free peptide AHRPam, which binds exclusively to βC holes,[22] provide insight into the specific binding of the knob–albumin conjugates. At a 20:1 molar ratio, it fully nullifies the inhibition effect of the GH–alb conjugate but only slightly weakens the inhibition by GP-alb. The results indicate that GH–alb is associating only with βC holes, but GP–alb associates with γC holes and, to a much lesser degree, βC holes, which tends to be the case with GPR free peptides.[3,4]

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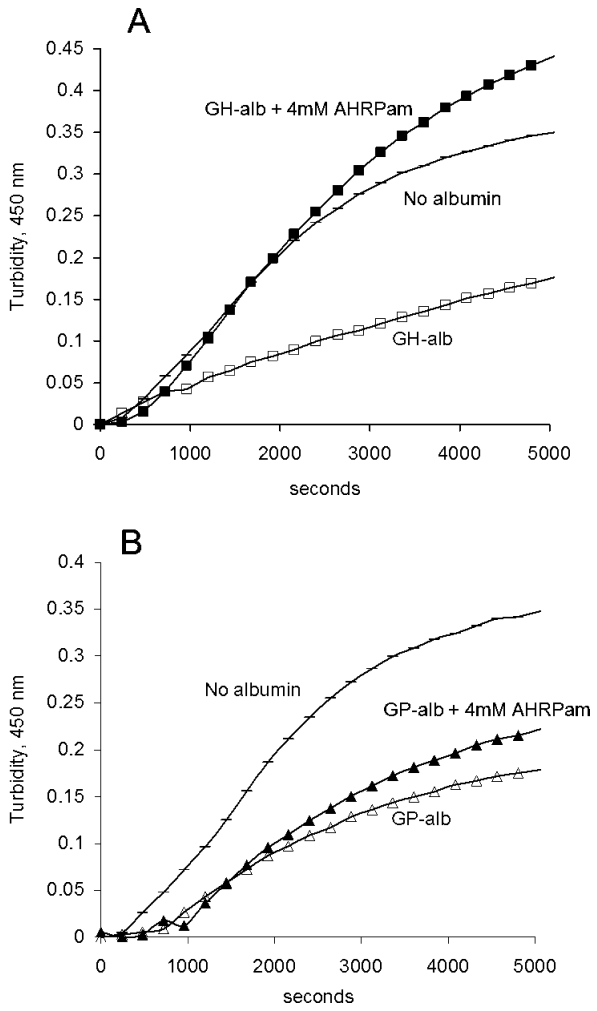

Figure 3. (A) High concentrations of the free AHRPam peptide completely nullify the inhibitory effect of GH–alb. Final concentrations were as follows: 210 μM GH–alb (□), 210 μM GH–alb with 4 mM AHRPam (■), and a reference (—). (B) In contrast, the same high concentration of AHRPam only slightly reduced the inhibitory effect of GP–alb. Final concentrations were as follows: 200 μM GP–alb (△), 200 μM GP–alb with 4.1 mM AHRPam (▲), and a reference (—). Reaction conditions as described in the legend of Figure 1.

In summary, this study demonstrates that synthetic peptides modeled on the A- and B-knobs of fibrin can be conveniently tethered to the solitary sulfhydryl of serum albumin with full retention of their capacity to interact with fibrin and fibrinogen. These peptide–albumin conjugates may prove to be useful in vivo because, unlike the free peptides, they should have long residence times in the circulation where they may modulate or slow fibrin formation. Because these peptide conjugates target fibrin polymerization directly, they may cause fewer side effects than clinical anticoagulants that target either the action or formation of thrombin.[23] On the other hand, they may interfere with fibrinogen–platelet interactions, the results of which may be untoward.

The question of whether the administration of exogenous albumin conjugates would result in concentrations sufficiently high to have any effect on fibrin polymerization in vivo arises. In fact, high concentrations of albumin are commonly administered for therapeutic purposes (shock, burns, coronary surgery, etc.), including situations in which unwanted clotting is

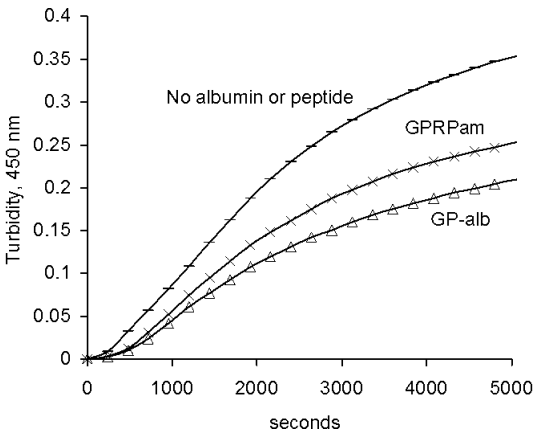

Figure 4. Model A-knob small peptide GPRPam inhibits fibrin polymerization and turbidity to degrees similar to that of the comparable concentration of GP–alb. Final concentrations were as follows: 200 μM GP–alb (70% substitution = 140 μM peptide) (△), 140 μM GPRPam (×), and a reference (—). Reaction conditions as described in the legend of Figure 1.

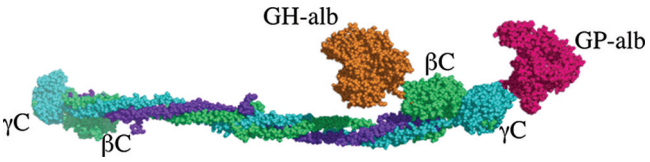

Figure 5. Hypothetical model of two peptide–albumin conjugates interacting with fibrinogen. GH–alb (brown) is bound to the βC hole and GP–alb (red) to the γC hole. Fibrinogen chains α (purple), β (green), and γ (blue).

a potential problem.[24] In situations of significant blood loss, replacement albumin conjugates would easily achieve concentrations that could moderate fibrin formation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Typical reaction course of peptides being linked to albumin by disulfide formation (Figure S1) and variation in turbidity profiles with different thrombin stocks (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

GPR, Gly-Pro-Arg; GHR, Gly-His-Arg; GPRPGGGGCam, Gly-Pro-Arg-Pro-Gly-Gly-Gly-Gly-Cys-amide; GHRPYGGGCam, Gly-His-Arg-Pro-Tyr-Gly-Gly-Gly-Cys-amide; GP–alb, GH–alb, and ae–alb, mixed disulfides of albumin with Gly-Pro-Arg-Pro-Gly-Gly-Gly-Gly-Cys-amide, Gly-His-Arg-Pro-

Tyr-Gly-Gly-Gly-Cys-amide, and 2-aminoethylthiol, respectively; AHRPam, Ala-His-Arg-Pro-amide.

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