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# Inactivator of the Third Component of Complement as an Inhibitor in the Properdin Pathway

**(glycine-rich β-glycoprotein/C3b/KAF/conglutinogen-activating factor/crossed electrophoresis)**

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**ABSTRACT** **Evidence has been obtained that a single protein, known to modulate classical complement activation, also acts as an inhibitor in the properdin or alternate complement pathway. A highly purified inactivator of the third component of complement (C3) from human serum inhibited the proteolysis of Factor B in the properdin system (glycine-rich β-glycoprotein) by glycine-rich β-glycoproteinase. The inhibition was by the enzymatic destruction of glycine-rich β-glycoproteinase activity. The major fragment of C3, C3b, which is the only known substrate of the C3 inactivator, blocked the destruction of glycine-rich β-glycoproteinase by the C3 inactivator. Thus, in its inhibition of the properdin pathway, the C3 inactivator destroys both the active form of glycine-rich β-glycoproteinase and a protein involved in the conversion of the zymogen form of this enzyme (proglycine-rich β-glycoproteinase) to its active form. The increased susceptibility to infections in a patient homozygous for deficiency of the C3 inactivator demonstrates the biologic significance of this protein.**

The complement (C) system consists of several serum proteins that, on activation, engage in sequential limited proteolysis, and thereby effect many reactions of the local inflammatory response. Activation of the third component of complement (C3) may occur either through the first three components of the "classical" pathway, C1, C4, and C2, or through a group of proteins comprising the properdin or "alternate" pathway. Three known inactivators brake and modulate the classical and common pathways at the C1 (1), C3, and C6 steps (2). The C3 inactivator is a proteolytic enzyme that destroys the hemolytic and immune-adherence activities of cell-bound, activated C3 (2) in the form of EAC $\overline{(1)4(2)3}$. This same protein may also act as conglutinogen-activating factor (KAF), converting bound C3 into a form that reacts with the bovine-serum protein, conglutinin (3). The C3 inactivator does not cleave native C3, but does act in the fluid phase on the major cleavage fragment of C3 produced by complement activation, C3b (4, 5).

A protein isolated from human serum has been designated glycine-rich β-glycoprotein (6), (GBG), and has the activity of Factor B (7) of the properdin system (8). This protein was subsequently isolated from normal human serum by Götze and Müller-Eberhard (9, 10), who termed it the C3 proactivator. An enzyme (GBGase) capable of cleaving GBG (11, 12) and destroying its activity as Factor B (8) was identified in the serum of a patient with a complex disorder (type I essential hypercatabolism of C3) affecting the properdin system and C3 (4, 13, 14). The same enzyme has recently been found in normal serum by Müller-Eberhard and Götze (15) and designated C3 proactivator convertase. An inhibitor of GBGase has been identified in normal serum (11, 12) and partially characterized as a 5–6S β-pseudoglobulin.

The present report provides evidence that the C3 inactivator also acts as GBGase inhibitor, that C3b inhibits this reaction, and that the patient with type I essential hypercatabolism of C3 is homozygous for an inherited deficiency of C3 inactivator.

## MATERIALS AND METHODS

*Preparation of GBG.* GBG was purified from fresh human plasma (6). Monospecific antiserum was produced in rabbits.

*Preparation of C3 Inactivator (KAF).* KAF was purified (3, and manuscript submitted to *Immunochemistry*) and monospecific antiserum was prepared in rabbits. The $F(ab)'_2$ fragment of purified antibody to KAF was made by pepsin digestion of KAF–anti-KAF precipitate.

*Protein Quantitations.* The concentrations of KAF, GBG, and C3 were measured in serum and reaction mixtures by electroimmunoassay (17) with monospecific antiserum or $F(ab)'_2$ from monospecific antiserum. KAF concentration was expressed as percent of that in a pool of serum from 50 normal persons.

*GBGase and Estimation of GBG Conversion.* Serum from the patient with type I essential hypercatabolism of C3 was used as a source of active GBGase (11, 12) in all experiments. This serum was devoid of detectable GBG (14) and KAF (4). It was used at a concentration of 33.3–50% in final reaction mixtures with GBG in a final concentration of 25 mg per 100 ml. All purified proteins were diluted with 0.15 M veronal-buffered saline (pH 7.4) containing 2 mM $Mg^{2+}$ unless otherwise indicated; this buffer was used in place of protein solutions in controls for each experiment. For the assessment of the influence of divalent cations on the action of KAF on GBGase, all reagents were dialyzed against 0.15 M veronal-buffered saline (pH 7.4) containing 10 mM EDTA and incubated at 37° for various times. The reaction mixtures were then dialyzed at 4° against the usual buffer containing $Mg^{2+}$

Abbreviations: C, complement; GBG, glycine-rich β-glycoprotein; KAF, conglutinogen-activating factor.

for assessment of GBGase activity. GBGase action on GBG was stopped at desired times by addition of EDTA to 10 mM and chilling at 4°. Antigen–antibody crossed electrophoresis (18) was used to estimate GBG conversion in essentially the same manner as was used for C3 conversion (19). The area of the GBG peak divided by the area of GBG plus the area of its $\alpha$-mobility conversion product multiplied by 100 gave the percent GBG remaining after various treatments. Fig. 1 shows some of the antigen–antibody crossed electrophoresis patterns.

*Chemical and Physical Treatments.* KAF was heated or treated with 2-mercaptoethanol, iodine, iodoacetamide, potassium periodate, or hydrazine (3). In addition, the effect of treatment of KAF for 10 min at 37° with soybean trypsin inhibitor (1 mg/ml), heparin (1 U/ml), tranexamic acid (1 mg/ml), and *p*-toluenesulfonyl L-arginine methyl ester (20 mM) was studied.

*Gel Filtration.* Gel filtration was on a 100 × 2.5-cm column of Sephadex G-200 in 0.15 M veronal-buffered saline (pH 7.4).

## RESULTS

*C3 Inactivator (KAF) as GBGase Inhibitor.* As shown in Fig. 2, prior incubation at 37° with KAF at a concentration 33% of that in normal serum for periods of 2, 5, or 15 min completely inhibited GBGase. The action of KAF on GBGase was not dependent on divalent cations since it proceeded effectively in 10 mM EDTA.

To determine whether KAF acted by stoichiometrically binding to GBGase or by enzymatically destroying GBGase, two experiments were performed. KAF at a concentration 50% of that in normal serum was incubated for 30 min at 37° with GBGase and filtered through Sephadex G-200. The resulting elution pattern was compared with that of KAF in normal serum. KAF emerged as an approximately 6S protein in identical relative elution positions in both instances, suggesting that its action on GBGase was not by formation of a long-lasting complex with the enzyme.

In a second set of experiments, KAF was incubated at 37°

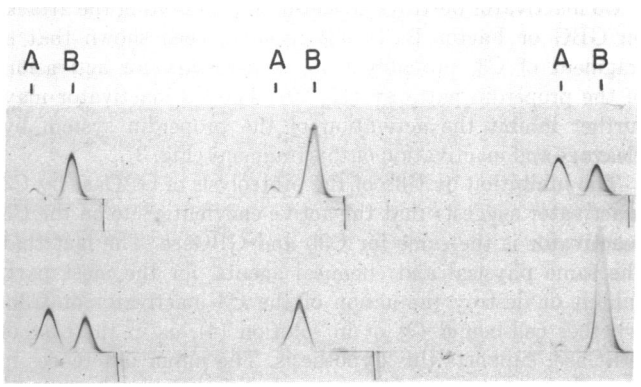


FIG. 1. Antigen–antibody crossed electrophoresis with antiserum to GBG. The anode for the first separation was at the left and for the second separation at the top. The positions of GBG(*B*) and its cleavage product, GAG(*A*), are indicated. GBGase was incubated at 37° with various concentrations of KAF for 2 hr followed by a second incubation with GBG added to a concentration of 25 mg/100 ml at 37° for 30 min. From left to right and top to bottom, KAF concentrations were 20, 10, 2, and 1% of that in normal serum; no KAF; and KAF at a concentration of 10% that in normal serum incubated with GBGase for 3 hr at 37°.

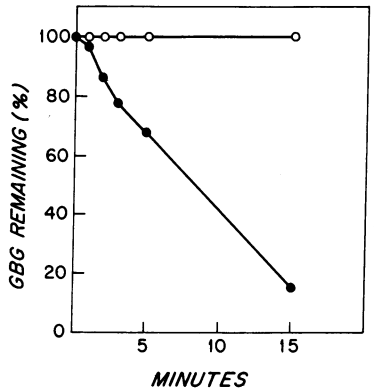


FIG. 2. GBGase action on GBG without KAF (●) or after prior incubation with KAF at a concentration of 33% that in normal serum for 2, 5, or 15 min (○).

at various concentrations and for various times with GBGase. As is evident from Table 1, KAF was fully effective in destroying GBGase at a concentration 0.2% of that in normal serum when incubated for 20 hr at 37°. GBGase in the absence of KAF was fully active against GBG when incubated under the same conditions. This suggested that the inhibition of GBGase by KAF was by proteolytic digestion.

Of the substances and treatments tested for their ability to inhibit the action of KAF on GBGase, soybean trypsin inhibitor, tranexamic acid, *p*-toluenesulfonyl L-arginine methyl ester, and hydrazine had no effect. Heparin at a concentration of 1 U/ml inhibited GBGase to some extent, but did not inhibit KAF action on GBGase. 2-Mercaptoethanol at a concentration of 10 mM was ineffective, but was inhibitory at a concentration of 100 mM. Potassium periodate (5 mM), iodine (1 mM), iodoacetamide (20 mM), and heating at temperatures of 54° or higher for 30 min partially destroyed the activity of KAF on GBGase.

TABLE 1. *Effect of KAF concentration and duration of prior incubation on GBGase activity**

| % KAF concentration | Prior incubation at 37° (hr) | % GBG remaining |
|---|---|---|
| 33 | 1 | 100 |
| 20 | 1 | 100 |
| 10 | 1 | 64 |
| 10 | 2 | 88 |
| 10 | 3 | 97 |
| 2 | 1 | 55 |
| 2 | 2 | 43 |
| 2 | 3 | 68 |
| 2 | 4 | 78 |
| 0.2 | 20 | 100 |
| 0 | 1 | 6 |
| 0 | 2 | 0.5 |
| 0 | 3 | 3 |
| 0 | 4 | 4 |
| 0 | 20 | 3 |

* GBGase was incubated at 37° with purified KAF at the concentrations given (as percent of normal serum concentration). GBG was then added to a concentration of 25 mg/100 ml, and the mixture was incubated for a further 30 min at 37°.

TABLE 2. *Inhibition of KAF action on GBGase* by C3b*

| C3b concentration (mg/100 ml) | % GBG remaining | % Apparent inhibition† |
|---|---|---|
| With KAF | | |
| 22 | 55 | 0 |
| 50 | 36 | 19 |
| 75 | 25 | 30 |
| 100 | 5 | 50 |
| Without KAF | | |
| 22 | 0 | — |
| 50 | 6.5 | — |
| 75 | 8.6 | — |
| 100 | 3.9 | — |

* GBGase was incubated for 1 hr at 37° with C3b and either KAF at a concentration 2% of that in normal serum or an equal volume of buffer (see *Methods*). GBG was added to a concentration of 25 mg/100 ml, and the mixture was incubated at 37° for an additional 30 min.

† Apparent inhibition was defined as percent GBG remaining in the test sample less that observed in the sample without added C3b (i.e., at a concentration of 22 mg/100 ml of C3b).

Since C3b in solution is a KAF substrate, it seemed possible that it is a competitive inhibitor of KAF in its action on GBGase. Accordingly, crude C3b was prepared by filtration of the patient's serum through Sephadex G-200; the serum contained about 22 mg of the C3 fragment per 100 ml and 8 mg of native C3 per 100 ml. Table 2 shows the effects of various concentrations of C3b on KAF inhibition of GBGase. It is apparent that C3b is an inhibitor of KAF action on GBGase. Incubation of GBGase with added C3b in the absence of KAF resulted in undiminished GBGase activity.

*KAF Concentrations in Serum of Family Members of the Patient with Type I Essential Hypercatabolism of C3.* Serum concentrations of KAF in the patient, his mother, six of his eight siblings, and several nieces and nephews are given in Table 3. His father was unavailable for testing. It is apparent that his mother, three of his siblings, and two nephews had

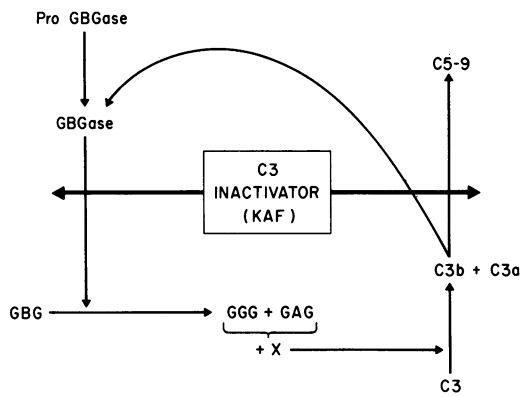


FIG. 3. Schematic representation of part of the properdin pathway and the points of action of the C3 inactivator as an inhibitor in the pathway. "X" represents as yet unidentified protein(s) mediating C3 conversion by one or the other GBG fragment. GAG and GGG are cleavage products of GBG; GAG is glycine-rich α-glycoprotein.

TABLE 3. *Concentrations of KAF, C3, and GBG in sera from the family of the patient with type I essential hypercatabolism of C3*

| Subject | KAF concentration (% nl) | C3 concentration (mg/100 ml) | GBG concentration (mg/100 ml) |
|---|---|---|---|
| Patient | <0.01 | 8 | <0.25 |
| *Mother | 46 | 126 | — |
| *Brother 1 | 48 | 140 | 58 |
| *Nephew 1 | 52 | 96 | 40 |
| *Nephew 2 | 42 | 104 | 46 |
| Nephew 3 | 99 | 104 | 40 |
| Nephew 4 | 164 | 164 | 87 |
| *Sister 1 | 46 | 134 | 83 |
| Niece 1 | 116 | 118 | 43 |
| Brother 2 | 75 | 118 | 43 |
| Sister 2 | 105 | 126 | 56 |
| Brother 3 | 132 | 134 | 37 |
| *Sister 3 | 38 | 118 | 27 |
| Normal range (Mean ± 2 SD) | 54–146 | 100–200 | 12–56 |

* Presumed heterozygous deficient for C3b inactivator deficiency.

KAF concentrations 50% of that in normal serum. Except for the patient, C3 and GBG concentrations were normal in all family members including those with a KAF concentration 50% of that in normal serum.

## DISCUSSION

These observations provide evidence that the C3 inactivator (KAF) (2, 3) is the same as GBGase inhibitor (11, 12). It is apparent that this enzyme not only attacks cell-bound (2) and fluid-phase (4, 5) C3b, thereby inhibiting the action of C3 on later-acting complement components, but it acts in two ways to inhibit the properdin or "alternate" pathway.

C3 inactivator destroys GBGase, thus inhibiting the attack on GBG or Factor B. It has recently been shown that a fragment of C3, probably C3b, is necessary for activation of the properdin pathway (15, 20). The C3 inactivator may further inhibit the activation of the properdin system by cleavage and inactivation of this fragment (Fig. 3).

The inhibition by C3b of the proteolysis of GBGase by C3 inactivator suggests that the active enzymatic site on the C3 inactivator is the same for C3b and GBGase. The fact that the same physical and chemical agents, for the most part, inhibit or destroy the action of the C3 inactivator on C3b, whether cell-bound (3) or in solution (4), as in the case of GBGase, supports this hypothesis. The minor differences in the effects of these agents may be attributed to differences in the sensitivity of the respective assays.

There have been difficulties in explaining the multiple abnormalities, which are seemingly unconnected, in the patient with Type I essential hypercatabolism of C3(4, 13, 14), although it has been shown by Nicol and Lachmann that many of his abnormalities could be mimicked *in vitro* by the immunochemical depletion of KAF from normal human serum (ref. 20 and *Immunology*, submitted for publication). The present observations provide a unifying explanation for

several of these abnormalities; it is now clear that the patient's primary defect is a genetically determined deficiency of C3 inactivator that acts in what is probably its biologically most important role as GBGase inhibitor. The failure to inactivate C3b and GBGase results in GBGase activation and uninhibited destruction of GBG. The generation of cleavage products of GBG, together with as yet unidentified additional serum protein(s), leads to inactivation and hypercatabolism of C3.

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