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Modified Crossed Immunoelectrophoresis to Study with Whole Plasma the Reversible Complex Formation of Histidine-Rich Glycoprotein with Plasminogen

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Key words

Plasminogen – Histidine-rich glycoprotein – Crossed immunoelectrophoresis – Binding assay – α_2-antiplasmin – Fibrinolysis.

Summary

To study the reversible complex formation between the plasma protein histidine-rich glycoprotein (HRG) and plasminogen, crossed immunoelectrophoresis of HRG was modified. In the modification, purified plasminogen was introduced into the gel of the first dimension electrophoresis.

Two molecular forms of plasminogen, Glu- and Lys-plasminogen, induced a dose-dependent reduction of the electrophoretic mobility of HRG, with a half maximal retardation for both plasminogens at 0.50–0.55 μM of added plasminogen to the agarose gel. HRG in plasma behaved as a uniform fraction with respect to plasminogen binding. In contrast, with the same modified technique another plasma protein, α_2-antiplasmin, separated into a retarded plasminogen-binding form and a non-retarded non-plasminogen-binding form.

The method can be used to assess several aspects of reversible complex formation between plasma proteins, as demonstrated for plasminogen binding of HRG and α_2-antiplasmin in whole plasma.

Introduction

Histidine-rich glycoprotein (HRG) is a protein which was originally discovered by Haupt and Heimburger (1, 2) in protein purification studies of plasma. Later it was determined in platelets as well (3). It has been found that HRG binds to heparin (2, 4), metal ions (5), plasminogen (6), thrombospondin (7) and fibrinogen (8) and inhibits autorosette formation (9). In fibrinolysis, the reversible binding of HRG to plasminogen results in inhibition of clot lysis due to the reduced availability of plasminogen (6).

Only immunochemical methods for the quantitation of the non-enzymatic HRG in plasma are described. At present no direct assays for the plasminogen binding of HRG are available. In several studies (10–16), the extent of binding of plasminogen to HRG has been assessed by calculation. From the total amounts of both proteins, the amount of non-complexed or "free" plasminogen has been calculated using the dissociation constant of the complex determined by Lijnen et al. (6), in studies with purified components. In such a calculation, normal binding to plasminogen is assumed but not tested for each plasma sample and genetic or acquired defects in plasminogen binding will not be traced. Additionally, it might be possible that more molecular forms of HRG, differing in plasminogen binding, exist in plasma. This would then be similar to that which has been established for α_2-antiplasmin another plasminogen binding plasma protein (18).

It can further be questioned whether the binding affinity determined with purified components is valid for the proteins in plasma. Thus, for the original determination of the dissociation constant between HRG and plasminogen, Lijnen et al. (6) used a degraded 60 kD purified form of HRG; later another larger molecule (80 kD) could be purified from plasma (9). Although the 80 and 60 kD molecules were subsequently found to exhibit similar plasminogen binding properties (17), this illustrates the above point that purification might introduce in principle artifacts. Another question concerns the possibility of occurrence of different molecular forms of HRG in plasma. This topic was raised in a report of Jacobsson et al. (19) who indicated that for binding to kringle 1 of plasminogen, two forms of HRG in plasma appear to exist: one binding, one non-binding.

This prompted us to devise a direct method to assess the binding of plasmatic HRG to plasminogen in order to address the question of the possible occurrence of HRG in multiple forms with respect to plasminogen binding and to be able to test individual samples for plasminogen binding without purification of the HRG. For this study, we adapted a modified crossed immunoelectrophoresis technique for whole plasma, previously used for studies on α_2-antiplasmin in plasma (18). It is further proposed that the principle of the modified crossed immunoelectrophoresis is suitable for study with whole plasma for reversible complex formation between other proteins as well.

Materials and Methods

"Agarose for electrophoresis" was obtained from BDH Chemicals Ltd., Poole, England. Plasminogen was prepared from human Cohn fraction III (kindly supplied by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) by affinity chromatography on lysine agarose (20) followed by gel filtration on Sephadex G-150 (Pharmacia Ltd., Uppsala, Sweden). Glu- and Lys-plasminogen were separated on DEAE-Sepharose (Pharmacia Ltd., Uppsala, Sweden) and quantified by using A 280 (1%) = 16.1 and a molecular weight of 90,000. Antiserum raised in rabbits against α_2-antiplasmin was a gift of Dr. I. Clemmensen, Copenhagen. Antiserum to HRG was raised in rabbits and its identity established by comparison with an antiserum kindly provided by Dr. N. Heimburger, Behringwerke AG, Marburg, West Germany. Pooled citrated plasma was obtained by mixing fresh platelet poor plasmas of 15–20 apparently healthy volunteers.

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis was performed essentially as described by Weeke (21). The first dimension was run for 3 h at 100 V; the second overnight at 100 V at ±10° C.

In the modified method, the 1% agarose gel for the first dimension in 0.03 mol/l sodium diethylbarbiturate/HCl, pH 8.6, contained plasmino-

Abbreviations:
HRG, histidine-rich glycoprotein; MCIE, modified crossed immunoelectrophoresis; HMr, high molecular weight.

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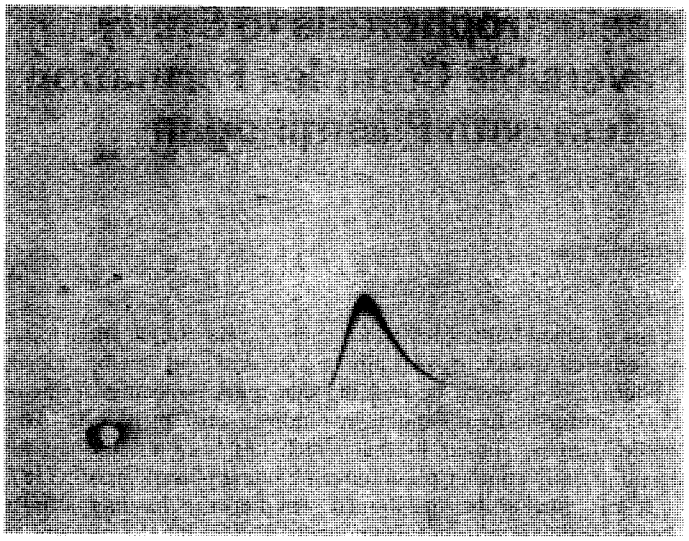

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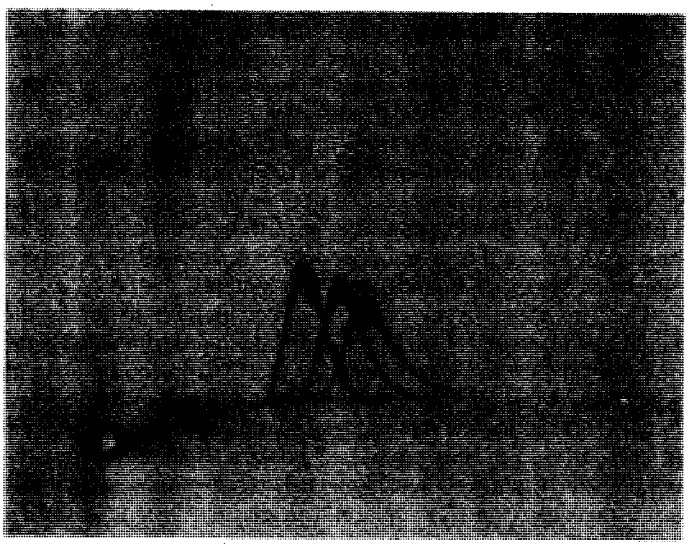

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Fig. 1 Normal and modified crossed immunoelectrophoresis for HRG. A) Top panel: Normal crossed immunoelectrophoresis for HRG. B) Lower panel: Photograph of three separate gels: during photography gels were superimposed. The peaks from right to left represent runs with no, 0.64 μM or 3.0 μM Glu-plasminogen, respectively in the gel of the first dimension

gen added to the agarose solution (±56° C) just before pouring the gel. The gel for the second dimension contained 0.5% antiserum against HRG. For the experiment shown in Fig. 3 A a mixture of 0.5% antiserum against HRG with 1% antiserum against α_2-antiplasmin was used.

For standardization purposes, several first dimension gels were run under identical conditions on one glass plate. Gel strips were cut and subsequently transferred to other glass plates for the second dimension electrophoresis.

Results

Modified Crossed Immunoelectrophoresis for HRG

With normal crossed immunoelectrophoresis using monospecific antiserum for HRG, HRG in plasma is manifested as a single peak with α_2-mobility, as illustrated in Fig. 1 A.

We modified the crossed immunoelectrophoresis by addition of plasminogen to the gel strip of the first dimension, thus providing a homogeneous concentration in the whole strip. When, after the first dimension electrophoresis (3 h at 100 V) the second dimension electrophoresis was performed with antiserum to plasminogen, a precipitation line parallel to the first dimension gel strip was obtained for nearly the whole length of the strip (not shown). This demonstrated that a homogeneous concentration of plasminogen was obtained and maintained during electrophoresis. This is in agreement with the low electrophoretic mobility of plasminogen under the conditions employed.

In the present study, we added Glu- or Lys-plasminogen to the first dimension gel in various concentrations. Fig. 1 B shows a superimposed photograph of three experiments with first dimension gels containing 0, 0.64 or 3.0 μM Glu-plasminogen. It can be observed that HRG shows a reduced mobility in the presence of the Glu-plasminogen and that the mobility reduction is dependent upon the plasminogen concentration. As shown in Fig. 2, the mobility reduction reaches an optimum of 26% at high Glu-plasminogen concentrations (above 2 μM). We observed similar results using Lys-plasminogen, with a closely similar mobility reduction of 28%. The half maximal mobility change for both Glu- and Lys-plasminogen was closely similar at 0.50–0.55 μM (cf. Fig. 2).

As can be observed from Fig. 1, the HRG remains as a single peak in the presence of Glu-plasminogen (and also in the presence of Lys-plasminogen, cf. Fig. 3 A). This is in contrast to α_2-antiplasmin which, under such conditions, shows two peaks, one retarded plasminogen-binding and one non-retarded non-plasminogen binding. As shown in Fig. 3 A, under conditions with excess of Lys-plasminogen, α_2-antiplasmin separates into two forms, while HRG remains as a single (retarded) peak. It is therefore concluded that plasminogen binding of HRG, in contrast to α_2-antiplasmin, is uniform.

The studies above were performed with pooled normal plasma (15–20 donors). In plasmas of 10 individual donors we further confirmed approximately half maximal retardation of HRG by approximately 0.5 μM Glu-plasminogen as one single peak.

Modified Crossed Immunoelectrophoresis for α_2-Antiplasmin

Thusfar, with the modified crossed immunoelectrophoresis, only the two molecular forms of α_2-antiplasmin were demonstrated for binding to Lys-plasminogen (18). The half maximal mobility change was found at 0.4 μM (23). In view of the reported high dissociation constant for purified α_2-antiplasmin and Glu-

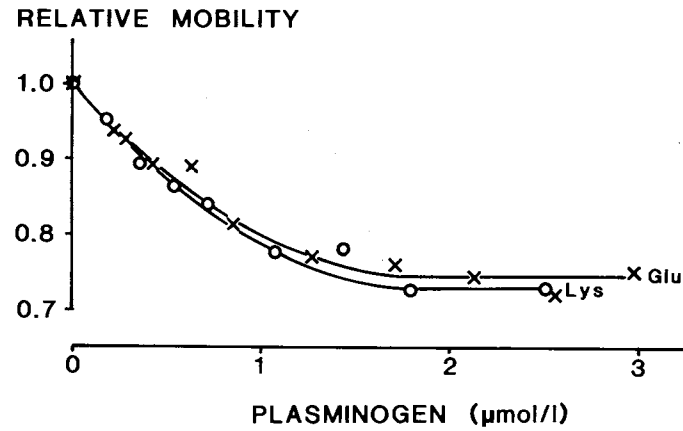


Fig. 2 Relation between retardation of the electrophoretic mobility of HRG and the Glu- (x) or Lys-plasminogen (o) concentration (abscissa) in the gel of the first dimension electrophoresis. The ordinate records the electrophoretic mobility of HRG relative to the mobility in the absence of added plasminogen (= 1)

plasminogen of 4.0 μM (24) we used 3.0 μM Glu-plasminogen in the gel and could show a separation of α_2-antiplasmin into two forms with Glu-plasminogen as well (Fig. 3B).

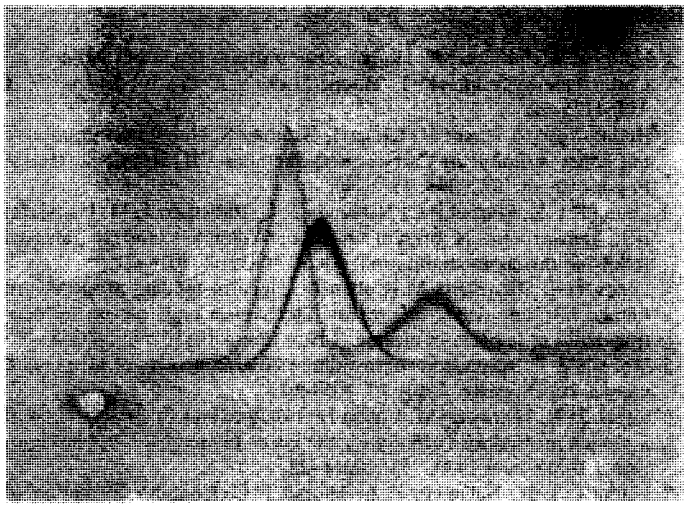
Discussion

We previously introduced a modification of crossed immunoelectrophoresis to separate the plasminogen and non-plasminogen binding forms of α_2-antiplasmin. In this method, the complex formation between α_2-antiplasmin and Lys-plasminogen is allowed in the first-dimension electrophoresis gel by incorporating a homogenous concentration of Lys-plasminogen into this gel (18). This complex formation can be visualized due to the difference in electrophoretic mobilities between free α_2-antiplasmin and the retarded α_2-antiplasmin-plasminogen complex. In the present study, we extended the use of this method to complex formation between plasminogen and HRG. We further studied the effects of different molecular forms of plasminogen and studied the retardation or complex formation as a function of the plasminogen concentration. The method appeared more generally applicable to study reversible complex formation between plasma proteins. In principle, this modified crossed immunoelectrophoresis has the advantage of using total plasma which does not require purification of the protein under study. The method can be used to demonstrate at least three aspects of reversible complex formation.

Firstly, it is possible to decide whether or not a reversible complex formation between proteins occurs, provided that the two molecules show a difference in electrophoretic mobility. We could demonstrate such complex formation for HRG and the two forms (Glu and Lys) of plasminogen. Further, complex formation between part of the plasma α_2-antiplasmin was now also demonstrated for Glu-plasminogen, in addition to previous demonstration for Lys-plasminogen (Fig. 3B).

Secondly, it is possible to decide whether or not the protein under study is uniform with respect to complex formation. In the present study, we have found that in contrast to α_2-antiplasmin, the binding of HRG to both Glu- and Lys-plasminogen appears as uniform. There is no significant difference in mobility change induced by Glu- and Lys-plasminogen. This is in agreement with the only slightly lower electrophoretic mobility of the Lys- versus the Glu-form of plasminogen (22). Our results are apparently different from those of Jacobsson et al. (19) who showed that plasmatic HRG was not uniform in its binding to isolated kringle I separated off from plasminogen. About 70% of the HRG was not retarded by sepharose bound kringle I (19); the reason for the difference is not clear. However, the difference in affinity of HRG to immobilized kringle I is, in our experiments, apparently not expressed in the binding of the intact plasminogen molecules (Glu- or Lys-plasminogen) to the HRG.

Thirdly, it is possible to study with the modified immunoelectrophoresis technique the affinity of the two reactants, as we did for plasminogen and HRG (cf. Fig. 2). With excess plasminogen, the maximal possible mobility change is to the position of the plasminogen-HRG complex and the half maximal mobility change represents an apparent dissociation constant of the complex. We found a value of 0.50–0.55 μM, close to the dissociation constant of 1.1 μM determined by Lijnen et al. (6) with purified proteins. It is now possible to compare the apparent dissociation constants for HRG in various plasma samples, notably for plasma samples of patients with acquired or congenital abnormalities of HRG (25). For the plasminogen-binding form of α_2-antiplasmin and Lys-plasminogen we determined, with the same technique, a value for half maximal retardation of 0.4 μM (23) which is close to the dissociation constant determined with purified components (K_d = 0.63) reported by Wiman et al. (24).



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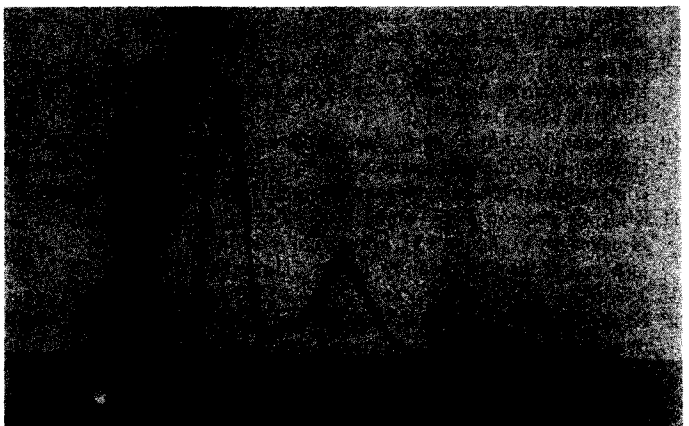

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Fig. 3 A) Top panel: Modified crossed immunoelectrophoresis of α_2-antiplasmin and HRG in the presence of excess (2.0 μM) Lys-plasminogen. The second dimension gel contains both an antiserum towards α_2-antiplasmin and towards HRG. The double peak represents α_2-antiplasmin. B) Lower panel: Modified crossed immunoelectrophoresis of α_2-antiplasmin in the presence of 3 μM Glu-plasminogen

Here, the method was applied to compare normal α_2-antiplasmin and a dysfunctional molecule α_2-antiplasmin Enschede (23).

We conclude that the modified crossed immunoelectrophoresis is a useful method for studying the reversible complex formation between plasma proteins, notably plasminogen with HRG or α_2-antiplasmin. It has the advantage that whole plasma can be used, thus avoiding effects of selective purification of putative multiple molecular forms in plasma. It also avoids complications of in vitro conversions of the molecule during purification methods.

The method can be applied to screen for variations in plasminogen binding by HRG or α_2-antiplasmin in cases with a suspected genetic variant and in diseases such as those with potential proteolytic modification of HRG (26). Further, application of the method can be suggested for other complexes, such as those between prekallikrein and HMr kininogen or factor XI and HMr kininogen (27), Thrombospondin and HRG (7), Protein S and complement factor C4Bb (28), heparin with antithrombin (29, 30), tetranectin (31) or HRG (4) and heparin with proteins (32).

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