Isoelectric Focusing of Human von Willebrand Factor in Urea-Agarose Gels

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An analytical technique has been developed for the isoelectric focusing (IEF) of plasma von Willebrand factor (vWF) in agarose gels containing urea. Under these conditions, vWF freely enters the gel and focuses without artifact. The focused vWF is visualized by staining fixed gels with 125I-labeled affinity-purified heterologous antibody. Utilizing a pH gradient of 5.0-6.5, normal vWF in plasma or purified preparations focuses into at least three bands with apparent isoelectric points (pI) between pH 5.7 and 5.9. A reproducible difference in the IEF pattern of vWF has been established between normal plasmas and those of individuals with variant von Willebrand's disease (vWd) type IIA and type IIB. In type IIA, vWF has a distinctly lower pI than normal. This difference may be related to the presence of smaller vWF multimers in IIA plasma because forms of vWF of corresponding size contained in normal cryoprecipitate supernatant have a similar pI. Type IIB von Willebrand factor has a pI intermediate between normal and IIA. Neuraminidase treatment of plasma samples before IEF results in an increase in pI in normal, type IIA, and type IIB vWF. The data suggest that none of the 16 type IIA and 9 IIB plasmas studied here contain significantly decreased amounts of sialic acid.

NORMAL PLASMA

von Willebrand factor (vWF) is a large glycoprotein aggregate composed of disulfide-bonded multimers that have been estimated to range in mol wt from approximately 800,000 to over 12 x 10^6. Structurally abnormal plasma vWF is present in variant vWd and lacks the larger multimers. In type IIA von Willebrand's disease (vWd), only the five smallest vWF multimers are usually present, whereas in type IIB, the intermediate size multimers can also be demonstrated. In addition to size differences, Gralnick et al. have found a reduced amount of carbohydrate in vWF purified from six patients with variant vWd. The sialic acid content of this abnormal vWF was reduced to 38%, 47%, and 41% of normal in the three individuals in whom it was specifically measured. Whether this reduced carbohydrate content is a generalized phenomenon in vWd has not been established.

We have now used isoelectric focusing (IEF) in urea-containing agarose gels to demonstrate charge differences between these pathologic molecules and normal vWF. The effect of releasing sialic acid from vWF was also examined. The urea-agarose IEF technique was developed because it was apparent that conventional agarose gel IEF of vWF resulted in numerous artifacts, probably attributable to the extremely large size of this molecule and its tendency to aggregate with other plasma proteins.

MATERIALS AND METHODS

Samples

Frozen citrated plasma was prepared as previously described. Purified vWF was prepared from fresh plasma by cryoprecipitation and agarose gel filtration and contained approximately 500 arbitrary U/dl of antigen by quantitative immunoelectrophoresis. An arbitrary unit is the amount of antigen present in 1 ml of a normal plasma pool.

Urea-Agarose Isoelectric Focusing

IEF was performed using an LKB 2117 Multiphor apparatus for flat bed IEF (LKB Instruments Inc., Los Angeles, Calif) equipped with an LKB 2103 constant wattage power supply. Agarose and ampholytes were also from LKB.

Agarose IEF gels were prepared according to LKB directions and stored overnight at 4°C in an air-tight container. For IEF in a wide pH range, the ampholyte composition was 70% pH 3.5-10, 10% pH 2.5-4, 10% pH 7-9, and 10% pH 9-11, using a total ampholyte concentration of 2%. For IEF in a narrow pH range, the ampholyte composition was 20% pH 3.5-10, 10% pH 3.5-5, 40% pH 4-6, 30% pH 5-7, 0.2% glutamic acid, and 0.2% glycine, using a total ampholyte concentration of 3%.

Prepared agarose IEF gels were placed in a sealed plastic box containing 50 ml of a solution composed of 7 M urea, 10% sorbitol, and the same mixture and concentrations of ampholytes that were used in the gel. The gels were equilibrated in this solution overnight at 4°C with gentle shaking.

Before use, excess urea solution was removed from the gels by draining them vertically and wiping the plastic backing with a damp cloth. The gels were not blotted. A single gel was placed on the Multiphor apparatus equilibrated at 10°C using a circulating waterbath. Kerosene was used to form a seal between the gel and cooling plate. Twenty-microliter samples were pipetted into the wells. All samples were diluted as indicated in this range of pH's.

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the same urea solution used to equilibrate the gel. The electrode strips were prepared and applied as directed by LKB, using 0.5 M acetic acid at the anode and 0.5 M sodium hydroxide at the cathode. The Multiphor electrode lid was used without a pressure bar.

The electrical conditions used for IEF were modified from those described by Rosen et al. For wide pH range IEF, first 20 min at a maximum of 100 V and 5 mA; then 60 min at a maximum of 200 V and 10 mA; and finally 90 min at 6.25 W. For narrow pH range IEF, the same conditions were used except that the final wattage was increased from 6.25 W to 20 W and the time from 90 min to 150 min. If necessary, focusing was briefly interrupted to carefully blot the cathode region of the gel with filter paper to remove accumulated moisture.

At the end of the focusing period the power was turned off and the cathode region of the gel was carefully blotted. The pH of the gel was determined at 1.0-cm intervals between the anode and cathode using an LKB surface electrode standardized in pH 7.0 reference buffer at 10°C. The electrode was rinsed and blotted between pH measurements. After pH determination, the electrode lid was replaced and the gel was refocused for 15 min at the final wattage setting. The electrode strips were removed and the gel was then placed immediately in a sealed plastic box containing 250 ml of 25% isopropanol, 10% acetic acid fixing solution for 1 hr.

The fixing solution and urea were then removed from the gel by placing the gel in a Kodak (Eastman Kodak Co., Rochester, N.Y.) slide hanger and washing it on a magnetic stirrer against 3 liters of distilled water for 1 hr with three changes of distilled water.

Visualization of vWF Using 125I-Labeled Antibody

The vWF was detected in the gels using 125I-labeled affinity-purified anti-vWF antibody raised in Australian emus. Purification of immunogen, immunization, affinity purification of antibody, and 125I labeling were as previously described for rabbit anti-vWF. The gels were reacted with the antibody as follows.

Washed gels were soaked for 30 min in PBS (phosphate-buffered saline, containing 0.02 M sodium phosphate, 0.15 M sodium chloride, and 0.02% sodium azide, pH 7.2) with 0.1% bovine serum albumin (BSA) (Fraction V, Sigma Chemical Co., St. Louis, Mo.). The gel was then placed in a sealed plastic box containing 0.2 mCi of 125I-labeled affinity-purified emu anti-vWF antibody (specific activity approximately 8 mCi/mg) which had been centrifuged 5 min at 7000 g in a table-top centrifuge and then diluted in 100 ml of the PBS/BSA buffer. The gel was incubated in this solution overnight at room temperature with gentle agitation on a rotary shaker. The antibody solution was reusable several times if it was stored at 4°C and bacterial growth prevented with sodium azide (0.02%).

After reaction with the antibody, the gel was washed as described above against 0.5 M sodium chloride and then dried and autoradiographed. Dried gels were also stained with Coomassie blue R250 and destained briefly in 10% ethanol.

Neuraminidase Treatment of Samples

Plasma and purified vWF samples were treated with neuraminidase using a modification of the method of Raum et al. Reaction mixtures consisted of 20 µl of plasma or purified vWF, 180 µl of buffer (PBS, 0.1% BSA, 10% 0.5 M citrate, pH 5.0, 0.02% sodium azide, final pH 5.1), and 10 µl of neuraminidase (Sigma type IX from Cl. perfringens reconstituted in PBS to 28.6 U/ml). Ten microliters of PBS replaced the neuraminidase in controls. The samples were incubated for 6 hr at 37°C and then overnight at 25°C. After centrifuging the samples 5 min at 7000 g in a table-top centrifuge, they were diluted 1:20 (plasma) or 1:50 (purified vWF) in the urea gel solution.

RESULTS

The Effect of Urea on IEF of vWF

The necessity for using urea to eliminate the interaction of vWF with other plasma proteins is illustrated in Fig. 1. When normal plasma was focused without urea, a large proportion of applied vWF remained at the point of sample application near the cathode and the rest closely approximated the pattern of the major plasma proteins, suggesting that vWF was associated with them. This was confirmed by the use of severe afibrinogenemic plasma in which the absent fibrinogen
band in the center of the gel coincided with an absent vWF band. This identified fibrinogen as one of the plasma proteins that associates with vWF in the absence of urea. Severe vWd plasma, which contains little or no vWF antigen, was used as a negative control. The usual major plasma proteins were present, but no vWF was detectable, thus demonstrating the specificity of the anti-vWF antibody. In the presence of urea, however, the normal plasma vWF completely entered the urea-containing gel and focused into a discrete series of bands. The vWF in the afibrinogenemic individual appeared identical to normal, demonstrating the lack of vWF-fibrinogen interaction.

Again, the severe vWd plasma revealed no detectable vWF. Therefore, the use of urea was necessary to prevent the interaction of vWF with other plasma proteins.

The Effect of Protein Concentration on Urea-Agarose IEF of vWF

A high dilution of plasma proved necessary to completely abolish the interaction of vWF with other plasma proteins (Fig. 2). When purified vWF was added back to vWF antigen-free plasma and focused at a 1:10 dilution (Fig. 2, left), the pattern resembled that of normal plasma focused at a 1:10 dilution. However,
these patterns were different from that of purified vWF focused alone, indicating that interactions between vWF and other plasma proteins still remained. When the plasma samples were diluted 1:200, purified vWF, normal plasma, and purified vWF added back to vWF antigen-free plasma all focused identically. Therefore, reducing the sample size to 0.1–0.2 μl of plasma was necessary to focus vWF without artifact.

Wide pH Range Urea-Agarose IEF of vWF in Normal and vWd Plasma

When vWF in normal plasma was compared with vWF in vWd type IIA plasma, a striking difference was seen. Type IIA vWF focused at a lower pH (5.4–5.7) than normal vWF (5.7) and the protein pattern was more diffuse (Fig. 3). When normal and type IIA plasmas were mixed, the vWF in each sample focused independently of the other (Fig. 3). Again, the severe vWd control plasma showed no detectable vWF antigen.

The lower molecular weight vWF, which remains in normal cryoprecipitate supernatant, focused similarly to type IIA vWF (Fig. 4). This suggests that the molecular size of the vWF aggregates influences its net charge, with lower molecular weight aggregates having lower isoelectric points.

To exclude the possibility that molecular sieving was occurring during IEF and to demonstrate that a steady state had been reached, samples were applied to the gel at different distances from the cathode and then focused. The results (Fig. 5) clearly demonstrated that an isoelectric steady state was reached and that the vWF moved freely in the gel, insuring that sample separations were based only on isoelectric point (pI). Thus, the large molecular size of normal vWF did not influence its pI determination in these gels.

Narrow pH Range Urea-Agarose IEF of vWF in Normal and vWd Plasma

When vWF was focused in a narrow pH range, the internal heterogeneity of normal plasma vWF was resolved and a more precise estimation of the pI of the various forms of vWF was possible. The narrow pH range gradient produced in these experiments ranged from pH 5.0 in the anode region to pH 6.5 in the cathode region. The pH gradients were determined for all gels run in this study and they were found to be highly reproducible (Fig. 6).

Both normal plasma vWF and purified vWF focused sharply into three major bands between pH 5.75 and 5.9 when a narrow pH range was used (Fig. 7). The vWd type IIA vWF was still diffuse and focused between pH 5.45 and 5.75 in the two different type IIA plasmas shown. The vWd type IIB vWF was also diffuse and different from both normal and type IIA, with a pI between the two. Thus, vWF in both types of vWd was different in IEF pattern from normal, with type IIB having a slightly lower pI and type IIA having the lowest pI.

During the course of this study, a total of 16 type IIA vWd plasmas, nine type IIB vWd plasmas, and 16 normal plasmas were examined. All were found to have IEF patterns and pls similar to those shown here. In addition, vWF in five hemophilia-A plasmas and in two serum samples examined appeared identical to normal. Eight severe vWd plasmas revealed little or no detectable vWF.
Urea-Agarose IEF of Neuraminidase-Treated vWF From Normal and vWd Plasma

Samples of purified vWF, normal plasma, vWd type IIA plasma, and vWd type IIB plasma were treated with excess neuraminidase as described. Control samples were incubated without neuraminidase. After incubation, the samples were diluted and focused in a wide pH range. Both normal plasma vWF and purified normal vWF shifted in pI from pH 5.7 to pH 6.1 after neuraminidase treatment (Fig. 8). Increasing the concentration of neuraminidase or extending the incubation time did not alter the results.

In vWd type IIA plasma, vWF also responded to neuraminidase treatment. The pI shifted from between 5.4 and 5.7 to between 5.7 and 6.1, indicating that sialic acid was present on this abnormal molecule (Fig. 8). In addition, the molecular heterogeneity was still present after sialic acid removal and the increase in pI of normal and vWd type IIA vWF was approximately equivalent. The desialylated type IIA vWF now focused at a higher pI than normal fully sialylated vWF. A similar shift in pI was seen in type IIB plasma after neuraminidase treatment.

DISCUSSION

Analytical IEF of plasma vWF in agarose gels has required the use of 7 M urea and sample sizes of 0.1–0.2 µl of plasma in order to allow vWF to focus freely and independently of other plasma proteins in the gel. However, the measurement of protein pI in the presence of 7 M urea requires two major qualifications. First, Uii has shown that 6 M urea increases the measured pI of ampholytes by 0.42 pH unit. Therefore, the pH measurements made here and the vWF pIs assigned must be lowered by at least 0.42 pH unit in order to compare them with IEF in the absence of urea. Second, the denaturing and disaggregating...
Fig. 7. Urea-agarose isoelectric focusing of von Willebrand factor from normal and von Willebrand's disease plasma in a narrow pH range. Samples of normal plasma (N), two different von Willebrand's disease plasmas type IIA (IIA), and one type IIIB (IIB) were focused. Pure von Willebrand factor was focused with similar plasmas in an identical gel and then aligned with normal plasma von Willebrand factor for comparison. The arrows indicate measured pH values. The anode is at the top. Plasma samples were diluted 1:100.

effect of 7 M urea on the structure of vWF will also influence its net charge and pl determination.

The finding that vWF in vWd type IIA focuses similarly to the vWF remaining in normal cryoprecipitate supernatant suggests that low molecular weight aggregates of vWF have a more negative net charge than high molecular weight aggregates. This is further supported by the observation that vWF in vWd type IIB, which contains both small and intermediate sized multimers but none of the largest multimers, has a pl intermediate between type IIA and normal. Thus, aggregation appears to reduce the number of surface negative charges. The similarity between normal cryoprecipitate supernatant vWF and IIA vWF is congruent with the findings of Nachman et al., who reported identical peptide maps for normal and type IIA vWF. The reason for the resolution of only normal vWF into sharply focused bands, while vWd types IIA and IIB and normal cryoprecipitate supernatant remain diffuse, is not known.

The use of excess neuraminidase to desialylate plasma and purified vWF samples was based on the method of Raum et al. Although the reaction mixtures in this experiment were prepared at pH 5.0,
which is optimal for the enzyme, it was found that the reaction could also be performed at pH 7.0 overnight at 37°C with similar results. The approximately equivalent increase in pI of normal plasma, purified vWF, and types IIA and IIB plasma vWF after neuraminidase treatment has important implications for the question of carbohydrate content of vWF in vWd. Neuraminidase treatment of type IIA and IIB vWF increased its pI to above the pI of normal vWF. It is therefore unlikely that any of the 16 type IIA and 9 IIB plasmas studied here contains a major deficiency of sialic acid and, by implication, of the neutral sugars to which it is attached. If sialic acid were significantly reduced in any of these type IIA and IIB vWd plasmas, their vWF would be expected to focus at a pI equal to or greater than normal vWF prior to neuraminidase treatment and treatment with the enzyme should have little or no effect on their pI.

The urea-agarose IEF technique has provided important information about the charge properties of the protein and carbohydrate components of normal and vWd vWF and should prove useful in future analysis of the structure of this complex multimeric aggregate. In addition, the urea-agarose IEF technique described here should be applicable to other large aggregated proteins for which vWF is a prototype.

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