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CHARACTERIZATION OF CHEMICALLY CROSSLINKED HUMAN FACTOR VIIIa

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The activity of factor VIIIa was enhanced and stabilized by treatment of factor VIII with the crosslinker disuccinimidyl suberate. The activity was >200-fold higher compared with that of native factor VIIIa and was stable for at least 15 days at 4°C and pH 7.2. The crosslinked factor VIIIa was purified by immunoaffinity chromatography and gel filtration. Electrophoretic analysis revealed high-molecular-mass (≈150 kDa) molecules as well as the three bands characteristic of native factor VIIIa. Thus crosslinking appeared to yield molecules stabilized by intra- and/or inter-subunit crosslinks. The material was further fractionated using immobilized von Willebrand factor and the factor VIIIa activity could be ascribed to trimers containing only intra-subunit crosslinks. Moreover, reduction of crosslinked factor VIIIa produced using dithiobis(succinimidylpropionate) suggested that the molecules containing inter-subunit crosslinks had not been cleaved by thrombin at arginine 372.

Factor VIIIa (fVIIIa) that has been activated by thrombin consists of three subunits (1-3). The A1 domain from the heavy chain is linked via a divalent metal ion to the light chain (A3C1C2) from which an NH_2-terminal 41-amino-acid activation peptide has been cleaved off. In addition, the A2 domain of the heavy chain remains associated with A1/A3C1C2 through an unknown mechanism. The liberation of the activation peptide releases fVIIIa from von Willebrand factor (vWf), whereas the thrombin cleavage between the A1 and A2 domains activates the cofactor function of fVIII (4). FVIIIa activity rapidly decays as the A2-A1/A3C1C2 complex dissociates (1-3). Crosslinking has been

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Abbreviations: fVIII(a), (activated) factor VIII; vWf, von Willebrand factor; DSS, disuccinimidyl suberate; DSP, dithiobis(succinimidylpropionate); BS^3, bis(sulfosuccinimidyl)suberate; F25, anti-A2 domain monoclonal antibody; ESH8, anti-C2 domain monoclonal antibody; MAb 18, anti-(1649-1689) monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; APTT, activated partial thromboplastin time.

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used to stabilize fVIIIa activity (5). The effect was attributed to intra-chain crosslinks since no high-molecular-weight molecular species was detected by electrophoresis under denaturing conditions. At the time of that crosslinking study, the subunit composition of fVIIIa and the cause of the decay of fVIIIa activity were not known. In a reinvestigation of the nature of the active form of crosslinked fVIIIa we have, in addition to the previously described form (5), obtained evidence for the presence of molecular species with an apparent mass of ≥150 kDa. This report describes the characterization of the different forms of crosslinked fVIIIa and the identification of the active molecular species.

MATERIALS AND METHODS

Reagents and standard methods. Recombinant two-chain B-domain-deleted fVIII was purified as described (6, 7) and vWf was isolated from plasma by adsorption of the vWf-fVIII complex on anti-vWf antibodies followed by elution of fVIII with 0.35 M $CaCl_2$ and subsequent elution of vWf with 3 M NH_4SCN. Thrombin (EC 3.4.21.5) was obtained from Boehringer Mannheim. Disuccinimidyl suberate (DSS), dithiobis(succinimidylpropionate) (DSP), and bis(sulfosuccinimidyl) suberate (BS^3) were from Pierce. The Superose 12 HR10/30 column, S-Sepharose Fast Flow and CNBr-activated Sepharose 4B were from Pharmacia-LKB Biotechnology. F25 (anti-A2 monoclonal antibody) and vWf were coupled to CNBr-Sepharose using standard procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 9% gels and silver staining were performed as described (8, 9). The silver staining background was reduced by the use of Farmer's reducer (Tetenal Photowerk). Protein transfer to nitrocellulose membranes (Schleicher & Schuell) was carried out on a semi-dry electroblotter (Ancos). Western blotting was performed using the antibodies F25 (30 μg/ml), ESH 8 (30 μg/ml) and MAb 18 (1 μg/ml) as primary antibodies and alkaline phosphatase-conjugated rabbit anti-mouse Ig (DAKOPATTS) as detection antibody. MAb 18 was a gift from Dr J. G. Gilles, Allergy and Clinical Immunology Unit, Institute of Cellular and Molecular Pathology, Université Catholique de Louvain, Brussels, Belgium. ESH 8 was from Bioscot Ltd., and F25 is an in-house monoclonal antibody. Automated activated partial thromboplastin time (APTT) reagent and fVIII-deficient plasma were from Organon Teknika and fVIII(a) activity was measured by an APTT assay, performed according to the manufacturer on an ACL 300 Research coagulometer (Instrumentation Laboratory).

Crosslinking and activation of fVIII. Crosslinking of fVIII (500-1800 units/ml) in 20 mM Hepes, pH 7.2, containing 0.1 M NaCl, 5 mM $CaCl_2$, 10% (v/v) glycerol, and 0.02% (v/v) Tween 80, was carried out by adding DSS or DSP (25 mM in DMSO) to a final concentration of 0.5 mM and incubating at 0°C for 10 min. Alternatively, BS^3 was added to a final concentration of 5 mM. Lysine was then added to a final concentration of 10 mM to quench excess crosslinker, followed by incubation at 0°C for 30 min. FVIII was activated with thrombin (0.2 units/ml) in the above buffer at 37°C for 30 min. FVIIIa crosslinked with DSP was reduced in electrophoresis sample buffer containing 20 mM dithiothreitol.

Purification of crosslinked fVIIIa activity. After activation by thrombin, crosslinked fVIIIa was applied to a 2.5-ml column of F25-Sepharose equilibrated

with 20 mM Tris-HCl, pH 7.3, 0.15 M NaCl, 10 mM $CaCl_2$, 0.02% (v/v) Tween 80 (buffer A). The column was washed with buffer A and the flow-through was collected. Bound protein was eluted with 50 mM imidazol, pH 7.3, containing 2.5 M NaCl, 10 mM $CaCl_2$, 50% (v/v) ethylene glycol, and 0.02% (v/v) Tween 80. The bound fraction was desalted on a NAP-25 column (Pharmacia) equilibrated with buffer A and concentrated to a volume of 300 µl using a Centricon-10 (Amicon). A 100-µl portion of the concentrated F25-Sepharose eluate was gel-filtered on a Superose 12 HR10/30 column equilibrated with 20 mM Hepes, pH 7.2, 1 M NaCl, 0.1 M lysine, 2.5 mM $CaCl_2$, 10% (v/v) glycerol, 0.01% (w/v) insulin, 0.02% (v/v) Tween 80 at a flow rate of 0.2 ml/min. The fractions that contained the highest fVIIIa activity were pooled and 50 units of this material was transferred to 20 mM imidazol, pH 7.3, containing 0.1 M NaCl, 10% (v/v) glycerol, 10 mM $CaCl_2$, and 0.02% (v/v) Tween 80, and incubated for 3 hours with vWf-Sepharose. The slurry was packed in a column, the gel was washed with the buffer, and bound protein was eluted with buffer containing 0.35 M $CaCl_2$.

RESULTS AND DISCUSSION

Crosslinking of fVIII with DSS prior to thrombin activation increased the stability of fVIIIa activity. One hour after thrombin activation, crosslinked fVIIIa was 200-400-fold more active than native fVIIIa. Crosslinked fVIIIa had around 10-fold higher activity than that of fVIII prior to thrombin activation, whereas unmodified fVIIIa had an activity that was approximately 5% of that of fVIII (Fig. 1). The activity of crosslinked fVIIIa was stable for at least 15 days at 4°C and pH 7.2.

It is noteworthy that treatment with DSS did not affect the biological activity or stability of fVIII, i. e. a difference was only observed with the thrombin-activated proteins. In an attempt to further increase the activity of crosslinked fVIIIa, the incubation time with DSS was prolonged or the water-soluble analog of DSS, BS^3, was employed to permit the use of increased concentration of crosslinker (5 mM). Longer incubation time had no effect, whereas an

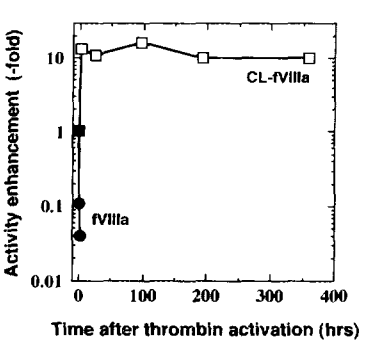

Figure 1. The activity of crosslinked fVIIIa (CL-fVIIIa) and fVIIIa as a function of time after thrombin activation. The activity of fVIII prior to activation was arbitrarily set to 1.

increased concentration of crosslinker had detrimental effects on fVIIIa activity. It should be mentioned that DSS and BS^3 were equally effective at 0.5 mM. Upon passage of crosslinked fVIIIa through a column of F25-Sepharose, more than 90% of the fVIIIa activity bound to the antibody and could be eluted with ethylene glycol. The activity in the flow-through may stem from fVIIIa molecules where the epitope for F25 had been modified beyond recognition by the crosslinker. When the same separation was repeated with native fVIIIa, no significant activity could be found in the flow-through nor in the eluate, probably due to complete dissociation of the native A2-A1/A3C1C2 complex. The fraction of crosslinked fVIIIa that bound to F25-Sepharose was further chromatographed on a Superose 12 column. FVIIIa peak activity eluted after approximately 0.55 column volumes.

The peak activity fraction was analyzed by SDS-PAGE and Western blotting. In addition to the bands corresponding to the light chain, A1, and A2, bands corresponding to molecular weights of ≥150,000 were visible (Fig. 2). This roughly corresponds to the mass of the fVIIIa heterotrimer. It thus appeared as if both inter- and intra-subunit crosslinks were formed. These results are not entirely compatible with those of an earlier study, where no evidence for inter-subunit crosslinks were obtained and hence no molecules with a molecular weight of more than 80,000 were observed (5). However, the use of two-chain B-domain-deleted recombinant fVIII in this study and full-length plasma-derived fVIII in the previous study may explain the different crosslinking patterns. In Western blotting, several bands could be detected with the anti-A2 antibody F25 (Fig. 2). In addition, two bands around 150 kDa and the free light chain reacted with an anti-C2 antibody (ESH 8). On a third blot (MAb 18), the activation peptide (light chain residues 1649-1689) was observed in the 150-kDa bands.

The bands around 150 kDa represented molecules that contained the A2 and C2 domains and had a mass corresponding to that expected of the fVIIIa

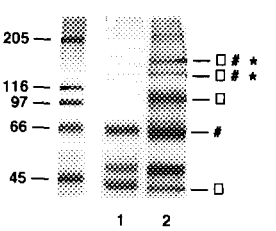

Figure 2. SDS-PAGE of fVIIIa (lane 1) and crosslinked fVIIIa (lane 2) on a 9% gel after sample reduction. The bands that were positive in Western blotting using F25 (□), ESH 8 (#), and MAb 18 (*) are denoted. The outer left lane shows molecular mass markers (kDa).

trimer. The activation peptide may be present due to lack of cleavage at arginine 1689 or it may remain crosslinked to the complex after thrombin cleavage. However, thrombin cleavage at arginine 1689 is not essential for fVIIIa activity in the absence of vWf (4). The presence of bands corresponding to the light chain, A1, and A2 indicated the existence of active fVIIIa trimers stabilized by intra-subunit crosslinks. The presence of residues 1649-1689 in the high-molecular-weight material suggested that it could be separated from the trimers stabilized by intra-subunit crosslinks using vWf affinity chromatography. Indeed, using this approach we showed that the high-molecular-weight material was selectively bound to vWf-Sepharose (Fig. 3). The bound fraction contained less than 10% of the fVIIIa activity of the starting material, whereas 90% of the activity was recovered in the flow-through. Moreover, this residual activity in the flow-through fraction presumably stemmed from the presence of contaminating trimer composed of non-covalently associated A1, A2, and A3C1C2 (Fig. 3, lane 3). Thus the fVIIIa trimers stabilized by intra-subunit crosslinks appeared to be responsible for the vast majority of, if not all, fVIIIa activity. The mechanism by which intra-subunit crosslinks stabilize the A2-A1/A3C1C2 complex is not known. One possibility is that crosslinking of fVIII locks the domains in a conformation that is normally not preserved after thrombin activation, and that this structure increases the affinity of A2 for A1/A3C1C2. This is supported by the observation that the stabilization of DSP-treated fVIIIa, but not that of DSS-treated fVIIIa, was abolished upon reduction (5).

Modification of fVIII with the reducible crosslinker DSP and activation by thrombin yielded a similar band pattern on SDS-PAGE as did DSS-treated fVIIIa (Fig. 4). Upon reduction of DSP-treated fVIIIa, concomitant with the disappearance of the high-molecular-weight material, a band appeared which probably contained intact fVIII heavy chain. This suggested that the molecules stabilized by inter-subunit crosslinks were not cleaved by thrombin between the A1 and A2 domains and supported the conclusion that they did not contribute to the fVIIIa activity of the crosslinked material.

A derivative of fVIIIa supported by covalent intra-subunit crosslinks with a long lifespan has obvious advantages over the native molecule whose activity vanishes only minutes after activation by thrombin. For instance, immobilized crosslinked fVIIIa, in spite of the fact that it has been modified, could be useful in studies of interactions between fVIIIa and other proteins. The increased stability may also be of pharmaceutical interest.

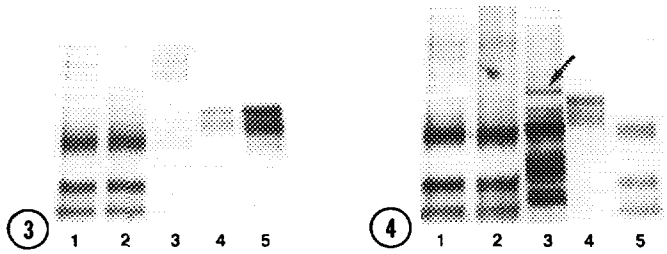

Figure 3. SDS-PAGE of pools from vWf-Sepharose chromatography of crosslinked fVIIIa and native fVIII on a 9% gel. Lane 1 is the applied crosslinked fVIIIa whereas lanes 2 and 3 show flow-through and eluate, respectively. Lanes 4 and 5 show the flow-through and eluate after application of fVIII. All samples were reduced.

Figure 4. SDS-PAGE of fVIIIa crosslinked with DSS or DSP on a 9% gel. Lanes 1 and 2 show unreduced fVIIIa modified with DSS and DSP, respectively. Lane 3 shows reduced DSP-treated fVIIIa and the arrow denotes the presumable fVIII heavy chain band. Lanes 4 and 5 show reduced fVIII and fVIIIa, respectively.

REFERENCES

1. Lollar, P., and Parker, C. G. (1990) *J. Biol. Chem.* 265, 1688-1692
2. Fay, P. J., Haidaris, P. J., and Smudzin, T. M. (1991) *J. Biol. Chem.* 266, 8957-8962
3. Lollar, P., and Parker, E. T. (1991) *J. Biol. Chem.* 266, 12481-12486
4. Hill-Eubanks, D. C., Parker, C. G., and Lollar, P. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 6508-6512
5. Fay, P. J. (1987) *Biochim. Biophys. Acta* 952, 181-190
6. Mikkelsen, J., Thomsen, J., and Ezban, M. (1991) *Biochemistry* 30, 1533-1537
7. Ezban, M., Chapman, B., Pedersen, J., Hansen, K., Rasmussen, P. B., Thomsen, J., Kjalke, M., and Kongerslev, L. (1993) *Thromb. Haemost.* 69, 1088 (abstract 1946)
8. Laemmli, U. K. (1970) *Nature* 227, 680-685
9. Morrissey, J. H. (1981) *Anal. Biochem.* 117, 307-310