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# AMINO GROUP MODIFICATION INHIBITS RISTOCETIN COFACTOR ACTIVITY OF HUMAN VON WILLEBRAND FACTOR

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SUMMARY: Purified von Willebrand factor rapidly loses activity when treated under mild conditions with the highly specific amino group reagent trinitrobenzenesulfonic acid. Greater than 90 percent inhibition of activity is achieved by modification of only 7 percent of the amino groups. Other modifications such as acetylation and succinylation also abolish activity. It is unlikely that the essential rapidly reacting amino groups function simply in an electrostatic manner since modifications such as amidination and methylation which produce derivatives which retain positive charge are also inactive or nearly so.

von Willebrand factor circulates in plasma as a heterogeneous series of polymers, composed of peptide chains of 230,000 daltons which are covalently linked by disulfide bonds (1-3). As part of the factor VIII complex von Willebrand factor is associated in plasma with another lower molecular weight peptide which carries factor VIII procoagulant activity. A number of observations have implicated von Willebrand factor in the adhesion of platelets to components of the vascular subendothelium which become exposed by endothelial injury (4-9). Relatively little, however, is known regarding detailed structural and functional correlations within this important molecule.

## MATERIALS AND METHODS

Materials - Trinitrobenzenesulfonic acid was obtained from Eastman, methyl acetimidate hydrocloride from Pierce Chemical Company and sodium borohydride, formaldehyde, acetic anhydride and succinic anhydride from Fisher Scientific. Ristocetin was purchased from Helena Laboratories. Sepharose 4B and cyanogen bromide activated Sepharose 4B were obtained from Pharmacia. Gelatin was prepared by heat denaturing purified lathyritic rat skin acid soluble collagen (10) in a boiling water bath. Other chemicals employed were of reagent grade.

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Purification of von Willebrand factor - von Willebrand factor was purified from the cryoprecipitate fraction of human plasma as described by Martin et al (11). The material was further purified by passage through a column of gelatin - Sepharose 4B (12) to remove contaminating fibronectin as recently described (8). The properties of the purified von Willebrand factor were as described earlier (8).

Assay of von Willebrand factor activity - von Willebrand factor was assayed as ristocetin cofactor activity as described by MacFarlane et al (13) using formalin-fixed, washed human platelets.

Quantitation of amino groups - The fraction of unmodified amino groups remaining after chemical modification was determined by comparison of the $A_{340}$ of equimolar amounts of control and amino group modified von Willebrand factor after reaction with 33 mg/100 ml trinitrobenzenesulfonic acid (final concentration) for 2 hr at 40° in the dark as described by Habeeb (14) for the quantitative estimation of amino groups. When trinitrobenzenesulfonic acid was the modifying reagent, the fraction of amino groups modified was determined by comparison of the $A_{340}$ following reaction with trinitrobenzenesulfonic acid under the various conditions described below with the $A_{340}$ produced by the quantitative reaction conditions of Habeeb (14).

Amino group modification - Amino group modifications were performed on 100µg portions of von Willebrand factor (7.7 units of ristocetin cofactor activity). In all cases control samples were processed in a manner identical to modified specimens except for omission of the active agent. All samples were extensively dialyzed against 0.15M NaCl, 0.05M sodium phosphate (pH 7.4) prior to analysis of von Willebrand factor activity.

Trinitrophenylation was performed by treating a 200µl of von Willebrand factor in 0.15M NaCl, 0.05M sodium phosphate (pH 7.4) with 200µl 4 percent $NaHCO_3$ (pH 8.5) and 200µl of either 0.1 percent or 0.01 percent aqueous trinitrobenzenesulfonic acid in the dark at ambient temperature for 15-120 min. The reaction was terminated by the addition of 10µl of 1M Tris·HCl (pH 8.5).

Acetylation with acetic anhydride was performed as described by Fraenkel-Conrat (15). Succinylation was performed according to the method of Chu et al (16) except that succinic anhydride was added dissolved in dioxane. Amidination with methylacetimidate hydrochloride was performed according to the procedure of Wofsy and Singer (17). Reductive methylation was carried out as described by Means and Feeney (18).

## RESULTS

Effect of trinitrophenylation - The purified von Willebrand factor was subjected to chemical modification with the highly specific amino group reagent trinitrobenzenesulfonic acid. Total amino groups were modified by treating the factor with 33 mg/100 ml of the reagent for 2 hr at 40 degrees as described by Habeeb (14). Aliquots of the protein were also modified to lesser extents under milder conditions by reaction with either 33 mg/100 ml or 3.3 mg/100 ml of trinitrobenzenesulfonic acid (final concentration) at room temperature for 15-120 min. Reactions were

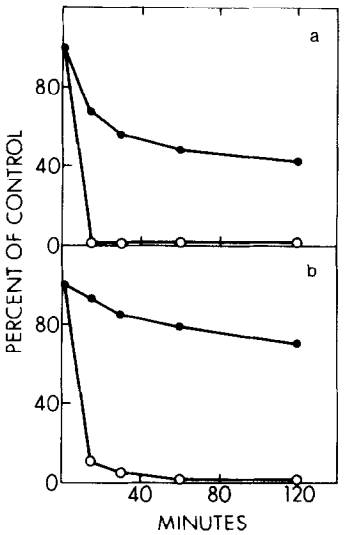


Fig. 1. Effect of trinitrophenylation on von Willebrand factor activity. 100µg of von Willebrand factor in 200µl of 0.15M NaCl, 0.05M sodium phosphate (pH 7.4) was treated with 200µl of 4 percent $NaHCO_3$ (pH 8.5) and 200µl of 0.1 percent trinitrobenzenesulfonic acid (a) or 0.01 percent trinitrobenesulfonic acid (b) at 23 degrees for 0-120 min. The reaction was terminated by the addition of 10µl of 1M Tris (pH 8.5). Following dialysis against 0.15M NaCl, 0.05M sodium phosphate (pH 7.4) ristocetin cofactor activity (o) and the unmodified amino groups (•) were determined. Modified amino groups were quantitated from the absorbance at 430 nm after the addition of 200µl 10 percent sodium dodecyl sulfate and 100µl of 1M HCl. Total amino groups were determined by reaction with trinitrobenzene sulfonic acid (200µl 0.1 percent) for 2 hr at 40 degrees.

terminated by the addition of excess Tris·HCl (pH 8.5). Following extensive dialysis against 0.15M NaCl, 0.05M sodium phosphate (pH 7.4), the fraction of total amino groups modified was determined spectrophotometrically from the absorbance at 430 nm. Ristocetin cofactor activity of control and modified von Willebrand factor preparations was determined from the rates at which the preparations agglutinated formalin fixed platelets in the presence of 1.5 mg/ml ristocetin.

When subjected to modification with 33 mg/100 ml trinitrobenzenesulfonic acid all biological activity was lost after 15 min of reaction. At this time 33 percent of the amino groups had been modified (Fig 1a). When a tenfold lower concentration of trinitrobenezenesulfonic acid was employed, 90 percent of the biological activity was lost by 15 min. At this time only 7 percent of the total amino groups had been modified (Fig 1b). After 60 min of reaction when all detectable biological activity was

TABLE 1

Effect of chemical modification of von Willebrand factor amino groups on ristocetin cofactor activity.

| Modification | Amino groups (percent modified) | Ristocetin cofactor activity (percent of control) |
|---|---|---|
| control | 100 | 100 |
| acetylation | 72 | nd[a](<0.3) |
| succinylation | 36 | nd (<0.3) |
| amidination | 24 | 14 |
| methylation | 67 | nd (0.08) |

100µg of purified von Willebrand factor was subjected to different amino group modifications as described under "Materials and Methods." After modification samples were dialyzed aainst 0.15M NaCl, 0.05M sodium phosphate (pH 7.4). The extent of modification was determined by reacting unmodified amino groups with trinitrobenzenesulfonic acid. Functional activity was assessed as ristocetin cofactor activity. All results are expressed relative to a control preparation for each modification which was subjected to treatment with a reagent blank.

[a] nd, not detectable, values in parentheses are upper limits based on the ability to detect 0.01U/ml ristocetin cofactor activity.

abolished only 20 percent of the amino groups were modified. Thus it appears that von Willebrand factor contains a class of rapidly reacting amino groups which are essential for activity.

Other amino group modifications - Trinitrophenylation results in the modification of amino groups with a large and bulky substituent. Furthermore, whereas the native amino groups are positively charged at physiologic pH, the modified amino groups carry no net charge. To further examine the amino group requirement of von Willebrand factor, a series of different amino group modifications was performed. The results are summarized in Table I. Acetylation which also converts a positively charged amino group to a neutral specie also eliminated all detectable activity.

After conversion of the amino groups to negatively charged derivatives by succinylation, the modified protein was again without biological activity. Two additional modifications were performed which resulted in derivatives in which the modified amino groups retained centers of positive charge. Reaction with methyl acetimidate produced a modified von Willebrand factor which retained approximately 14 percent of its original activity. However this reaction resulted in modification of only 24 percent of the total amino groups. Reductive methylation results in the conversion of primary amines to secondary and tertiary amines without displacement of the center of positive charge from the amine nitrogen. This rather subtle modification also resulted in the loss of all biological activity.

## DISCUSSION

Considering the important role von Willebrand factor plays in hemostasis, it is surprising that relatively little information exists regarding structural and functional correlations within this molecule. It is now well established that ristocetin cofactor activity is lost upon reduction of the interpeptide chain disulfide bonds (19-23). Galactose residues have been implicated in von Willebrand factor function (24-26). Conflicting results have appeared regarding a possible role for sialic acid (26-28).

The present study demonstrates that modification under mild conditions of rapidly reacting amino groups of von Willebrand factor with trinitrobenzenesulfonic acid results in elimination of biological activity. Greater than 90 percent of activity, expressed relative to a control preparation which was treated in an identical manner except for omission of the active agent, was destroyed by modification of only 7 percent of the von Willebrand factor amino groups. Based upon published amino acid analyses of human von Willebrand factor (41-44) there are 96-99 lysine residues per peptide chain. Thus modification of 6-7 of these residues results in an essentially inactive molecule.

Acetylation, which modifies amino groups with a less bulky substituent, also abolished activity. However, both acetylation and trinitrophenylation result in conversion of positively charged amino groups to neutral species. Succinylation, by which positively charged amino groups are converted to negatively charged derivatives, also resulted in inactive von Willebrand factor. Two modifications which produced modified amino groups that retained positive charge were also performed. Amidination modified 24 percent of the total amino groups and produced 86 percent inhibition of ristocetin cofactor activity. It is not clear if this slight retention of activity is due to the retention of positive charge by the modified protein, even though the charged center is displaced from its original position, or is due to a decreased reactivity and increased selectivity of methyl acetimidate. Reductive methylation which results in the formation of secondary and tertiary amines in which the positive charge is still centered on the original amine nitrogen produced a derivative with no detectable activity. These results suggest that these rapidly reacting amino groups are essential for von Willebrand factor activity. These amino groups presumably do not function simply in an electrostatic manner as both derivatives which retain positive charge were inactive or nearly so.

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