# Localization of Binding Sites within Human von Willebrand Factor for Monomeric Type III Collagen†

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ABSTRACT: Purified human plasma von Willebrand factor (vWf) binds to pepsin-digested monomeric type III collagen in a saturable ($K_D$ = 1 × $10^{-8}$ M), specific, and rapid manner with a stoichiometry of approximately 1:15 [vWf subunit ($M_r$ 270 000):collagen trimer ($M_r$ 300 000)]. Two reduced and alkylated CNBr peptides of vWf, termed M11 residues 542–622 and M20 residues 948–998 [Titani, K., Kumar, S., Takio, K., Ericsson, L. H., Wade, R. D., Ashida, K., Walsh, K. A., Chopek, M. W., Sadler, J. E., & Fujikawa, K. (1986) *Biochemistry 25*, 3171–3184], inhibited vWf binding to collagen. With $^{125}$I-vWf (2 × $10^{-9}$ M) as ligand, M11, M20, fragment III (a dimeric, V8 protease, $NH_2$-terminal fragment, $M_r$ 320 000 referenced above), and unlabeled vWf inhibited binding to collagen with $EC_{50}$ values of 4.8 × $10^{-7}$, 9.4 × $10^{-7}$, 1.1 × $10^{-7}$, and 0.2 × $10^{-7}$ M, respectively. M11 and M20 bind to collagen directly when $^{125}$I-labeled peptides are used as ligands. Other CNBr fragments of vWf were less effective as inhibitors (5-fold or less) and bound less avidly to collagen (5-fold or less) compared to M11 and M20. A murine anti-human vWf monoclonal antibody (MR5), which blocks the binding of vWf to collagen, bound selectively to both M11 and M20 when tested in an enzyme-linked immunoadsorbent assay. Fragments M11 and M20 contain a region of amino acid sequence homology, residues 597–621 and 969–992, and are encoded by repeat domains A1 and A3, defined by analysis of cDNA sequences for human vWf [Shelton-Inloes, B. B., Titani, K., & Sadler, J. E. (1986) *Biochemistry 25*, 3164–3171]. Human vWf contains two binding sites for monomeric type III collagen. The sites are located between residues 542–622 and 948–998 and may relate to regions of sequence homology shared by these portions of the vWf molecule.

Plasma von Willebrand factor (vWf)[1] is a multimeric glycoprotein composed of similar subunits, $M_r$ 270 000 (Legaz et al., 1973; Hoyer & Shainoff, 1980; Titani et al., 1986), that is synthesized by endothelium (Jaffe et al., 1973) and mediates platelet adhesion through successive interactions with vascular subendothelium and glycoprotein (GP) Ib of the platelet surface (Tschopp et al., 1974; Weiss et al., 1978; Sakariassen et al., 1979). Since vWf does not interact with the surface of unactivated platelets, the process of vWf binding to subendothelium appears to activate or alter vWf and promote its interaction with the platelet surface although no direct evidence is available for this activation step (Kao et al., 1979). Both intact subendothelium (Sakariassen et al., 1979) and subendothelial components such as collagen and microfibrils (Baumgartner et al., 1977; Fauvel et al., 1983) have been studied as examples of vascular sites for vWf binding and activation. In perfusion studies, collagen serves as a substrate for vWf-dependent platelet adhesion when present in the subendothelium or as a film on artificial surfaces (Houdijk et al., 1985). Direct studies of the vWf–collagen interaction indicate that both fibrillar collagen and monomeric collagen (Nyman, 1977; Scott et al., 1981; Bockenstedt et al., 1986) interact with the larger multimers of vWf (Kessler et al., 1984). Studies of monoclonal antibodies to vWf indicate that certain regions of the vWf molecule subserve collagen binding (Girma et al., 1986). Both a tryptic peptide, $M_r$ 48 000 (Sixma et al., 1984), and a subtilisin peptide, $M_r$ 23 000 (Girma et al., 1986), appear to serve as sites for interaction with collagen.

In this study, we characterize the interaction of vWf with monomeric type III collagen, and we delineate two regions of the vWf molecule that govern vWf binding to monomeric type III collagen.

## Experimental Procedures

*vWf.* Human plasma vWf was purified from pools of 25 type A plasma cryoprecipitates by using steps of recryoprecipitation, absorption with $Al(OH)_3$ and bentonite, precipitation with 4% (precipitate discarded) and 12% (precipitate used) poly(ethylene glycol), and gel filtration on Sepharose CL-4B (Pharmacia) (DeMarco & Shapiro, 1980; Miller et al., 1983). Protein concentration was determined by the method of Bradford (1976) with a bovine serum albumin (BSA) standard. By analysis on reduced 6% $NaDodSO_4$–polyacrylamide gel electrophoresis (PAGE), vWf preparations contained one predominant subunit ($M_r$ 220 000) and, in addition, 100–125 units/mg vWf antigen (Hoyer & Trabold,

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[1] Abbreviations: vWf, von Willebrand factor; CNBr, cyanogen bromide; GP, glycoprotein; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; ELISA, enzyme-linked immunoadsorbent assay; PBS, phosphate-buffered saline; BME, β-mercaptoethanol; IgG, immunoglobulin G.

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1982), 110–120 units/mg ristocetin cofactor activity, and a normal complement of multimers (Hoyer & Shainoff, 1980). Molar concentrations of vWf are based on the size of the vWf subunit, $M_r$ 270 000. For peptide preparation, vWf was purified from commercial factor VIII concentrate (Titani et al., 1986). Digestion with *Staphylococcus aureus* V8 protease was performed with a 1:100 ratio of enzyme:substrate, yielding fragments II (COOH terminal, $M_r$ 100 000 subunit) and III ($NH_2$ terminal, $M_r$ 170 000 subunit) which were isolated on a Mono Q column (Pharmacia). CNBr, tryptic, and *Achromobacter* protease (lysine enzyme) peptides were prepared from reduced and carboxymethylated vWf by using sequential gel filtration and reverse-phase HPLC steps to isolate individual peptides (Titani et al., 1986). vWf and vWf peptides were labeled with $^{125}I$ (Fraker & Speck, 1978), separating free and protein-bound $^{125}I$ by gel filtration on Sephadex G-25. $^{125}I$-vWf contained one major band, $M_r$ 220 000, as assessed by $NaDodSO_4$–PAGE and autoradiography.

For preparation of murine anti-vWf monoclonal antibodies, Balb/C mice were immunized twice with a 2-week interval with 20 μg of human vWf and given 150 μg of vWf intravenously 5 months later, 3 days prior to fusion of spleen cells with NSO myeloma cells (Kennet, 1980). Resulting hybridomas were screened with vWf-coated polystyrene wells and alkaline phosphatase conjugated goat anti-mouse IgG, recloned, expanded in tissue culture, and injected in pristane-primed mice. A series of ten anti-vWf monoclonal antibodies were prepared and shown to be non-cross-reacting by enzyme-linked immunoadsorbent assay (ELISA) noted above. IgG was isolated from ascitic fluid by $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography.

An ELISA for vWf and vWf peptides was developed. Antigens bound to microtiter wells (50 pmol in 0.05 mL, 20 mM $Na_2CO_2$, 16 h, 4 °C) were incubated in turn with 0.1% BSA, 20 mM $NaH_2PO_4$, pH 7.4, 0.14 M NaCl (BSA/PBS), murine monoclonal anti-vWf antibody in BSA/PBS, goat anti-mouse IgG–alkaline phosphatase conjugate, and phosphatase substrate, measuring absorbance at 405 nm ($A_{405}$). Between steps, wells were washed 3 times with 20 mM $NaH_2PO_4$, pH 7.4, 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.02% BME, and 0.05% Tween 20 (wash buffer). Monoclonal antibody, 60.5, directed against a leucocyte surface protein, served as control; control $A_{405}$ values were subtracted from test sample results.

*Collagen.* Native (Glanville, 1982) and pepsin-digested (Fujii & Kuhn, 1975) collagens were prepared from fetal calf skin and separated into type I and type III forms by differential salt precipitation (Miller & Rhodes, 1982). Samples were subjected to DEAE-cellulose column chromatography to remove acidic impurities and procollagen contaminants (Glanville, 1982). Collagen concentration was determined by hydroxyproline analysis (Kivirikko et al., 1967) and extrapolation from the reported content of hydroxyproline in type I and type III collagen (Miller, 1982). Each collagen preparation was greater than 95% pure as assessed by 5% $NaDodSO_4$–PAGE (Miller, 1982) and was iodinated with Bolton–Hunter reagent (Sage et al., 1981). Affinity-purified rabbit polyclonal anti-collagen antibody was prepared against both type I and type III pepsin-digested collagen (Furthmayr, 1982).

Collagen was bound to detachable plastic (Immulon I, Dynatech) microtiter wells (0–2.0 μg of collagen, 0.2 mL of 0.1 M acetic acid, 16 h, 4 °C) and washed 3 times with wash buffer. Collagen bound to plastic was measured by using both $^{125}I$-labeled collagen and ELISA assay, performed as described above for vWf with rabbit polyclonal anti-collagen antibody and goat anti-rabbit IgG–alkaline phosphatase conjugate. The binding of $^{125}I$-labeled collagen was diminished in the presence of unlabeled collagen, and the ELISA for bound collagen indicated that iodination had no effect on collagen binding to plastic. Most experiments were performed with wells containing 10 ng of bound collagen, requiring incubation with 600 ng of collagen in 0.2 mL.

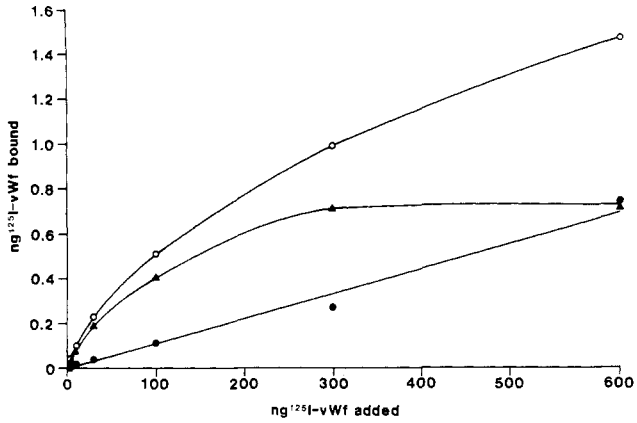


FIGURE 1: Binding of $^{125}I$-vWf to collagen. $^{125}I$-vWf was incubated with 10 ng of collagen, and bound vWf was measured as $^{125}I$ content (Experimental Procedures). Each point represents the mean of quadruplicate determinations with (○) $^{125}I$-vWf alone (total binding), (●) $^{125}I$-vWf plus 10 μg of unlabeled vWf, and (▲) the difference between total and nonspecific binding, termed specific binding (Limbird, 1986).

*Binding Assay.* Each well was incubated with collagen, washed once with wash buffer, and blocked with 0.2 mL of BSA/PBS (60 min, 22 °C). $^{125}I$-Labeled ligand (intact vWf, V8 (intact protease fragments or CNBr peptides), alone or in the presence of unlabeled proteins or peptides, was added in 0.05 mL of BSA/PBS. For antibody experiments, the $^{125}I$ ligand was preincubated (15 min, 22 °C) with antibody. Following incubation of the $^{125}I$ ligand with bound collagen, the well was aspirated, washed 3 times with wash buffer, separated, and assayed for $^{125}I$ content in a γ scintillation counter. In the presence of 10 μg of unlabeled vWf, any bound $^{125}I$ was considered nonspecific and was subtracted from test results. In wells without collagen, the amount of bound $^{125}I$-vWf was negligible. Collagen-bound $^{125}I$-vWf, which was eluted and subjected to $NaDodSO_4$–PAGE and autoradiography, contained one major radioactive protein, $M_r$ 220 000.

## RESULTS

The binding of $^{125}I$-vWf was dependent on both the amount and form of collagen present. With 400 ng of $^{125}I$-vWf as ligand, a progressively decreasing extent (2.4, 1.4, 0.6, 0.25 ng) of total vWf binding was observed with decreasing amounts (33, 10, 3.3, 1.0 ng, respectively) of pepsin-digested type III collagen. Compared to type I collagen, type III collagen was 3–4-fold more effective in binding vWf. With approximately 100 ng of collagen as substrate and 200 ng of vWf as ligand, type I collagen bound 1.75 ng of vWf while type III bound 5.5 ng. Native and pepsin-digested collagen of the same type, either I or III, bound similar amounts of vWf. Pepsin-digested type III collagen (10 ng bound to plastic) was used in all subsequent experiments.

As shown in Figure 1, vWf binds to monomeric type III collagen in a saturable manner with approximately 0.7 ng of vWf bound specifically at saturation to 10 ng of collagen, corresponding to an approximate stoichiometry of 1:15 [vWf subunit ($M_r$ 270 000):collagen trimer ($M_r$ 300 000)]. About 100 ng of vWf/0.05 mL ($K_D$ of $1 \times 10^{-8}$ M; vWf subunit, $M_r$ 270 000) gave 50% maximal vWf binding to collagen (Figure

Table I: vWf Peptides—Inhibition of vWf–Collagen Binding and Reactivity with Antibody MR5

| peptide[a] | % inhibition[b] | reaction with MR5, $A_{405}$[c] |
|---|---|---|
| vWf, 1–2050 | >92 | >1.00 |
| fragment III, 1–1365 | >92 | >1.00 |
| fragment II, 1366–2050 | 10 | 0.10 |
| M11, 542–622 | 71 | 0.28 |
| M20, 948–998 | 71 | 0.47 |
| CNBr group 1 | 20–41 | ≤0.05 |
| CNBr group 2 | 0–16 | ≤0.10 or NT |

[a] Ranges refer to residues of vWf (Titani et al., 1986); fragments III and II are V8 protease peptides; M11 and M20 are CNBr peptides; CNBr peptide group 1 includes M6, M9, M13, M14 and group 2 includes M5, M17, M18, M19, and M24 with $A_{405}$ ≤0.10 and M15, M21, M23, and M25–27 with $A_{405}$ not tested (NT). [b] Percent inhibition by 100 pmol of peptide of $^{125}$I-vWf (0.1 pmol) binding to collagen in the binding assay (Experimental Procedures). [c] Reaction of peptide with monoclonal antibody MR5 measured as absorbance at 405 nm ($A_{405}$) in ELISA (Experimental Procedures) with 50 pmol of peptide and 0.4 μg of MR5.

1), comparable to the concentration of vWf in normal human plasma of approximately $4 \times 10^{-8}$ M (vWf subunit, $M_r$ 270000). The observed binding reaction is specific for vWf. With 25 ng of $^{125}$I-vWf/0.05 mL ($2 \times 10^{-9}$ M; vWf subunit, $M_r$ 270000) as ligand, unlabeled vWf, fibrinogen, and fibronectin inhibited collagen binding with approximate $EC_{50}$ values of $0.02 \times 10^{-6}$, $4.0 \times 10^{-6}$, and $8.0 \times 10^{-6}$ M, respectively. The vWf–collagen interaction was rapid (80–90% complete within 30 min) and reversible (>80% loss of bound $^{125}$I-vWf after 60 min at 22 °C with a 50-fold excess of unlabeled vWf). Binding experiments were performed at 22 °C. No binding was observed at 37 °C, presumably due to denaturation of pepsin-digested collagen.

*vWf Peptides as Inhibitors of vWf–Collagen Binding.* The collagen-binding domain of vWf lies in the $NH_2$-terminal portion of the molecule, fragment III (Girma et al., 1986). CNBr cleavage of reduced and carboxymethylated fragment III results in 29 peptides of which 17 (see Table I) were studied and 12 were not (5 peptides, M2, M7, M22, M28, and M29, were unavailable and 7 peptides, M1, M3, M4, M8, M10, M12, and M16, contained 1–12 residues) (Titani et al., 1986). As shown in Table I, 100 pmol of vWf or vWf peptides was incubated with 0.1 pmol of $^{125}$I-vWf in 0.05 mL ($2 \times 10^{-9}$ M) for 10 min at 22 °C and then added to a binding assay. vWf, fragment III, and CNBr peptides M11, residues 542–622, and M20, residues 948–998, gave >50% inhibition under these conditions and gave the following $EC_{50}$ values with $2 \times 10^{-9}$ M $^{125}$I-vWf as ligand: vWf, $2 \times 10^{-8}$ M; fragment III, $1.1 \times 10^{-7}$ M; M11, $4.8 \times 10^{-7}$ M; M20, $9.4 \times 10^{-7}$ M. Other CNBr peptides (M6, M9, M13, and M14, group 1 of Table I) showed some ability to inhibit binding (20–41% compared to 71% for M11 and M20) but 5-fold less reactivity with the monoclonal antibody, MR5 (see results below). Mixtures of M11 and M20 did not show additive activity as inhibitors of the vWf–collagen interaction.

CNBr fragments M11 and M20 were also labeled with $^{125}$I and tested for their capacity to bind directly to collagen; M19, M24, and a mixture of all peptides showing inhibitory activity (Table I) were used as controls. Approximately 1.4% of added $^{125}$I-M11 and 0.5% of added $^{125}$I-M20 bound to collagen compared to 0.1% of control $^{125}$I peptides.

*Inhibition of vWf–Collagen Binding by Murine Anti-vWf Monoclonal Antibodies.* A series of ten non-cross-reacting, murine monoclonal antibodies to human vWf were evaluated as inhibitors of vWf binding to collagen. Antibodies were preincubated with vWf (100 ng of antibody, 100 ng of $^{125}$I-vWf, 10 min, 22 °C) and added to the binding assay. One antibody, termed MR5, gave 100% inhibition under these conditions. Antibodies MR4, MR1, MR11, MR8, and MR3 gave 2, 4, 10, 19, and 30% inhibition, respectively. Four antibodies (MR2b, MR6, MR9, and MR10) showed no inhibitory activity. Identical incubations using 10 ng of antibody resulted in 100% inhibition of vWf binding to collagen by MR5 and <10% inhibition by the other antibodies. With an ELISA technique, individual vWf peptides were bound to plastic microtiter wells and tested for their ability to react with MR5. The antigenic determinant for MR5 resides in fragment III but both M11 and M20 also appear to contain some portion of the antigenic determinant for this antibody. As shown in Table I, M11 and M20 gave higher absorbance readings (0.28 and 0.47, respectively) compared to both CNBr group I and CNBr group II peptides (≤0.05 and ≤0.10, respectively). To test if peptides in CNBr group I were capable of binding to plastic wells, M6, M9, M13, and M14 were tested by an identical ELISA technique using MR2b, MR3, MR8, MR11, and the control non-vWf monoclonal antibody, 60.5. Each peptide bound to plastic as shown by a distinctive pattern of reactivity with the individual antibodies. The lower levels of reactivity between MR5 and various CNBr peptides are unexplained, but similar low levels of reactivity were seen with each of the monoclonals tested. In addition to inhibiting the binding of vWf to collagen, MR5 also blocked the binding of vWf to microtome-cut sections of bovine aorta (Roth et al., 1983, and unpublished observations).

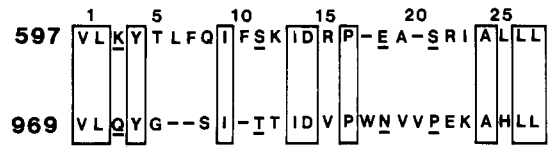


FIGURE 2: Regions of amino acid sequence homology within two CNBr peptides of vWf, M11 and M20. M11 includes residues 542–622 within the cDNA repeat domain A1, while M20 includes residues 948–998 within repeat domain A3 (Titani et al., 1986; Shelton-Inloes et al., 1986). Two gaps are inserted in the sequence of M11 and three in M20 to permit the ordering of amino acid residues. Identical residues are enclosed in boxes and conservative substitutions are underlined, being defined as those substitutions with positive values in the mutation data matrix of Dayhoff et al. (1983). Amino acids are denoted by the single letter code.

*Inhibition of vWf–Collagen Interaction with Smaller Peptides.* The above data suggest that M11 and M20 contain a region of the vWf molecule capable of interaction with collagen. Comparison of the primary amino acid sequence of the two fragments revealed regions of amino acid sequence homology, residues 597–621 of M11 and residues 969–992 of M20 (Figure 2). To determine if these regions are related to the vWf–collagen interaction, both tryptic and lysine enzyme fragments of M11 or M11+12 (a contiguous peptide of M11 and M12) were tested as inhibitors of vWf binding to collagen. The following tryptic fragments contain no activity as inhibitors of the interaction: residues 566–586 (YAGSVASTSEVLK), residues 609–616 (IDRPEASP), and residues 617–629 (IALLLMASQUEPQR). The lysine enzyme peptide, residues 609–622 (IDRPEASRIALLLM), possesses minimal activity (6% inhibition using 100 pmol of peptide and 0.1 pmol of $^{125}$I-vWf). Two chemically synthesized peptides, VLKY and VLQY, were inactive as inhibitors of the vWf–collagen interaction.

## Discussion

In vivo, vWf binding to collagen serves as an initial step in hemostasis and mediates platelet adhesion to the damaged vessel wall. The in vitro binding of vWf to collagen, described

in this study, has the appropriate properties of saturability, specificity, and speed (Limbird, 1986) and occurs at vWf concentrations similar to those of plasma.

As a representation of the vascular side of the binding reaction, type III collagen is severalfold more active than type I collagen, consistent with the localization of the type III form in subendothelium (Gay et al., 1975). However, the 1:15 stoichiometry (vWf subunit:collagen trimer) indicates that the binding site for vWf in collagen is unavailable in some trimers or requires multiple trimers for its expression. In addition to collagen, other structures in the subendothelium may also contain binding activity for vWf since anti-vWf antibodies that block the vWf–collagen interaction also block vWf binding to intact subendothelium (Sixma et al., 1984). Examples of such noncollagenous vWf binding sites may include proteoglycans (Wight, 1980) and microfibrils (Stemerman et al., 1971). Therefore, the collagen binding site of vWf may prove to be a more general "connective tissue" binding site, interacting with different affinities with several subendothelial components: colloquially, a "one fits all" type of binding site. Nevertheless, such a site may be studied, as described, by using a purified, highly reactive substrate such as monomeric type III collagen.

The data indicate that human vWf contains two binding sites for type III collagen, identifiable with CNBr peptides of vWf and anti-vWf monoclonal antibodies. Two peptides, M11 and M20, inhibit binding of intact vWf to type III collagen, bind to collagen themselves, and interact directly with a monoclonal antibody, MR5, which inhibits binding of vWf to collagen. The epitope for MR5 appears to be expressed more completely on M20 than on M11, while M11 retains more collagen binding activity than M20.

The peptides are obtained from reduced and alkylated vWf and are subjected to solubilization in 6 M guanidine, 80% formic acid, and acetonitrile. Therefore, the denatured form of these peptides, determined by their primary structure, appears to contain or contribute to the structural features necessary for binding to collagen. The peptides appear to share a common structure since they both interact with MR5 and since mixtures of the two peptides do not lead to an additive inhibitory effect on the vWf–collagen interaction. In addition, the peptides are translated by regions of vWf mRNA that show sequence homology as determined by the nucleotide sequence of the cDNA coding for the protein (Shelton-Inloes et al., 1986). The actual nature of the proposed common structure shared by M11 and M20 is unknown but could involve the regions of amino acid sequence homology shown in Figure 2. The striking feature of these regions is the presence of three similar tetrapeptides interrupted by stretches of 5–8 nonhomologous amino acids. The basic structure is

$$VL(K,Q)Y\text{–}ID(R,V)P\text{–}A(L,H)LL$$

with the residues in parentheses referring to single amino acid differences within the tetrapeptides of M11 and M20. However, in contrast to vWf binding to activated platelets, which is mediated by the tetrapeptide sequence RGDS (Plow et al., 1985), a single tetrapeptide sequence was not found to affect vWf binding to collagen. For example, peptides containing VLKY, VLQY, IDRP, or ALLL did not inhibit binding and CNBr peptide M17, which is encoded by the A2 domain and contains a VLQY sequnce but lacks IDVP and AHLL, was also inactive as a binding inhibitor.

In summary, human vWf contains at least two binding regions for type III collagen. The minimal extent of the polypeptide chain required for binding activity and the actual amino acid sequence involved remain areas for further study.

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## *Articles*

# Selective Deamidation and Enzymatic Methylation of Seminal Ribonuclease†

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ABSTRACT: Isoenzymatic forms $\alpha_2$, $\alpha\beta$, and $\beta_2$ of bovine seminal ribonuclease are generated by the transformation of $\beta$-type into $\alpha$-type subunit through deamidation of a single amide group [Di Donato, A., & D'Alessio, G. (1981) *Biochemistry 20*, 7232–7237]. The residue involved in this selective deamidation has been identified as $Asn^{67}$. Deamidation occurs by formation of a cyclic imide intermediate involving the Gly at position 68. Opening of the cyclic imide may occur on either side of the nitrogen, generating both the normal $\alpha$-aspartyl and an isoaspartyl residue at position 67. The $\alpha$-carboxyl of the isoaspartyl residue is effectively methylated by bovine brain protein carboxylmethyltransferase.

Bovine seminal ribonuclease (BS-RNase)[1] is a ribonuclease isolated from bovine seminal plasma and seminal vesicles (D'Alessio et al., 1972; Tamburrini et al., 1986). It is a dimer, with two disulfides and noncovalent forces linking together the two subunits (Di Donato & D'Alessio, 1973; D'Alessio et al., 1975). In an earlier paper (Di Donato & D'Alessio, 1981) it has been reported that (1) the enzyme, as isolated, is a mixture of three isoenzymic forms, $\alpha_2$, $\alpha\beta$, and $\beta_2$, (2) $\beta$-subunit transforms into $\alpha$-subunit upon hydrolysis of a single amide group, and (3) the production of isoenzymes has to be physiological, as the isoenzymes are consistently found, and in the same ratios, irrespective of the enzyme source (seminal plasma or seminal vesicle tissue) and of the isolation procedures. Two main questions were left unsolved: the identification of the selectively labile amide residue and the mechanism of deamidation.

We report here on the identification of the asparaginyl amide group, in the $Asn^{67}$-$Gly^{68}$ sequence, as the amide selectively hydrolyzed when $\beta$-subunit transforms into $\alpha$-subunit and on the mechanism of deamidation, involving a cyclic imide intermediate, which spontaneously hydrolyzes, producing either a normal aspartyl or an isoaspartyl residue. The finding that the free $\alpha$-COOH of the latter can be stoichiometrically methylated by the enzyme *S*-adenosylmethionine:protein carboxyl-*O*-methyltransferase (protein methylase II) provides a further example, after that of porcine adrenocorticotropin (Aswad, 1984; Murray & Clarke, 1984), on the involvement of deamidation at Asn-Gly sequences in the generation of methylatable sites in proteins. It may also provide a basis for an understanding of the possible physiological meaning of the selective deamidation leading to the transformation of $\beta$- into $\alpha$-subunit of BS-RNase. This in turn may be used as a suitable experimental system for studying the general significance of the production of methylatable $\alpha$-COOH groups in proteins.

## Experimental Procedures

*Selective Deamidation of BS-RNase.* BS-RNase, purified from bull seminal plasma or from seminal vesicles (Tamburrini et al., 1986), was deamidated at 37 °C in 0.2 M potassium phosphate, pH 8.4, at a protein concentration of 2 mg/mL (Di Donato & D'Alessio, 1981). The extent of deamidation was monitored by fast-protein chromatography (Tamburrini et al., 1986) of aliquots of the deamidation mixture. Under these conditions, a virtually complete transformation of heterogeneous protein into a homogeneous preparation of selectivity deamidated subform $\alpha_2$ was obtained. Purification of subforms was carried out by ion-exchange chromatography on (carboxymethyl)cellulose as described (Di Donato & D'Alessio, 1981).

*Preparation of Fully Reduced and Carboxymethylated Proteins.* Fully reduced and alkylated proteins were prepared

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[1] Abbreviations: BS-RNase, bovine seminal ribonuclease; RNase A, bovine pancreatic ribonuclease A; ACTH, adrenocorticotropin; CM, carboxymethyl; RP-HPLC, reverse-phase high-pressure liquid chromatography; $Na_2EDTA$, disodium ethylenediaminetetraacetate.

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