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Tetranectin, a trimeric plasminogen-binding C-type lectin

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Abstract

Tetranectin, a plasminogen-binding protein belonging to the family of C-type lectins, was expressed in *E. coli* and converted to its native form by in vitro refolding and proteolytic processing. Recombinant tetranectin—as well as natural tetranectin from human plasma—was shown by chemical cross-linking analysis and SDS-PAGE to be a homo-trimer in solution as are other known members of the collectin family of C-type lectins. Biochemical evidence is presented showing that an N-terminal domain encoded within exons 1 and 2 of the tetranectin gene is necessary and sufficient to govern subunit trimerization.

Keywords: C-lectin; domain structure; refolding; trimerization

Tetranectin (TN) is a homo-oligomeric Ca^{2+}-binding protein primarily found in plasma (10 mg/L) and in the extracellular matrix of certain human carcinomas, whereas little or no TN is found in the extracellular matrix of the corresponding normal tissues (Christensen & Clemmensen, 1989; 1991; Wewer & Albrechtsen, 1992). Although specific binding of TN to plasminogen kringle-4 domain (PLM K4), to complex sulfated polysaccharides (Clemmensen, 1989) and to proteins in the extracellular matrix have been shown (Kluft et al., 1989a, 1989b), indicating that TN may participate in processes involved in tissue remodeling, the physiological role of TN remains to be clarified.

The mature TN polypeptide chain, 181 amino acid residues with three intra-chain disulfide bridges (Fuhlendorff et al., 1987), is encoded in three exons as shown by molecular cloning and characterization of the gene (Berglund & Petersen, 1992; Wewer & Albrechtsen, 1992). The posttranslational modification of threonine-4, originally reported by Fuhlendorff et al. (1987), has been identified as O-linked glycosylation (Jaquinod et al., in prep.). On the basis of gel filtration data, natural human TN was originally proposed to occur as a homo-tetramer in solution (Clemmensen et al., 1986).

Exon 3 of the human TN gene encodes a separate functional and structural unit, a single long-form carbohydrate recognition domain (CRD) clearly related to C-type lectins by sequence homology, conservation of disulfide topology (Fuhlendorff et al., 1987) and by presence of an almost complete suit of amino acid residues predicted to be involved in Ca^{2+} and sugar binding. Due to the reported tetrameric nature along with single residue differences TN is considered to belong to a distinct class of C-type lectins (Drickamer & Taylor, 1993; Day, 1994).

In the present study, we report on cross-linking analysis of the homo-oligomeric organization of natural and recombinant TN in solution.

Results

A series of TN derivatives, rTN123 (E1–V181, mature protein), rTN12 (E1–V49, exon 1 and 2), rTN23 (V17–V181, exon 2 and 3), and rTN3 (A45–V181, exon 3), was expressed in *E. coli* and refolded in vitro with the purpose of analyzing the oligomeric nature of TN.

Construction of the rTN expression plasmids

By appropriate design of the 5′ PCR-oligonucleotide primer, the 543 bp reading frame encoding the mature human TN monomer (E1 to V181; amino acid numbering from Fuhlendorff et al. (1987) was extracted from a tetranectin cDNA clone and joined to a nucleotide sequence encoding the amino acid sequence GSIEGRG, containing a cleavage site for the plasma serine protease FX_a (FX_a) (Nagai & Thøgersen, 1987). To facilitate cleavage of the fusion proteins by FX_a a glycine residue was inserted in the P_1' position, between the P_1 arginine residue and the N-terminal glutamic acid residue of TN. The amplified DNA fragment was cloned into the *E. coli* expression vectors pT7H6 and pT7CIIH6 as described in Materials and methods. Plasmids for expression of the H6-rTN12, H6-rTN23, and H6-rTN3 fusion proteins were also constructed with N-terminal FX_a cleavage sites by amplification of corresponding DNA fragments using the appropriate oligonucleotide primers listed in Materials and methods and pT7H6-TN123 as template. A

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summary of rTN fusion protein constructs used in the present work is shown in Figure 1.

Expression, refolding, and processing of H_6-rTN fusion proteins

Each TN derived fusion protein was expressed in *E. coli* DH1 or BL21 cells, purified on Ni^{2+}-NTA-agarose, and in vitro refolded as described in Materials and methods. All rTN derivatives, except for rTN12, were liberated by FX_a cleavage and purified by ion-exchange chromatography on Q-Sepharose (not shown).

Characterization of recombinant human Tetranectin rTN

Natural and recombinant TN (rTN123) were compared in assays testing for binding to immobilized PLM K4, calcium, heparin, and reaction with a rabbit polyclonal anti TN antibody and found to be identical in terms of all functional criteria tested (not shown). Mass spectrometric analysis of tryptic peptides derived from rTN123 confirmed that the disulfide bridge topology of the recombinant protein was identical to that of the natural protein (Jaquinod et al., in prep.). Furthermore, structural analysis of recombinant TN (rTN123) and its CRD (rTN3) by crystallography is well in progress (Kastrup et al., 1996).

Chemical cross-linking analysis of the Tetranectin oligomeric structure

TN has been suggested to be a tetrameric protein of 68 kDa composed of four identical subunits of 21 kDa (Clemmensen et al., 1986). In the present study the stoichiometry of subunits in TN was analyzed by chemical cross-linking studies of human TN from plasma and of rTN123 (Fig. 2A). Cross-linking of natural TN or rTN123 with dimethylsuberimidate (DMSI) was found to produce three bands, i.e., monomer, dimer, and, apparently, trimer.

Determination of the molecular weight of an oligomer by SDS-PAGE analysis by chemical cross-linking is biased, because a significant number of residues (e.g., lysine when the protein is cross-linked with DMSI) react with the reagent thereby increasing the molecular weight of the complex and may, in addition, impose other structural changes to the protein influencing gel mobility.

To overcome this problem a combinational strategy based on the approach of Eisenstein and Schachman (1989), performing cross-linking studies of in situ generated hybrid complexes of rTN123 and the larger fusion protein CIIH6-rTN123 was developed (Fig. 2B). No detectable subunit exchange between pre-formed homo-oligomers in a mixture of rTN123 and CIIH6-rTN123 was found after 16 h at room temperature. Subunit exchange could be induced by incubating the protein mixture at 70 °C for 15 s before cooling to room temperature and addition of DMSI. SDS-PAGE analysis showed the presence of four trimer bands above 95 kDa (corresponding to two homo-trimers and two hetero-trimers) and three dimer bands (corresponding to two homo-dimers and one hetero-dimer) in the gel between 43 and 55 kDa, in relative abundance in agreement with random association of monomer subunits into trimers and dimers after subunit exchange. It should be noted, that molecular weight markers have only been included on the SDS-PAGE gels for crude calibration and orientation of the gels.

The trimeric organization of TN was further corroborated by cross-linking studies of the proteins H6-rTN12, rTN23, and rTN3 and mixtures between them (Fig. 3). The TN derivative rTN3, containing only the CRD, could not be cross-linked even at high protein concentrations and did not interfere with the cross-linking

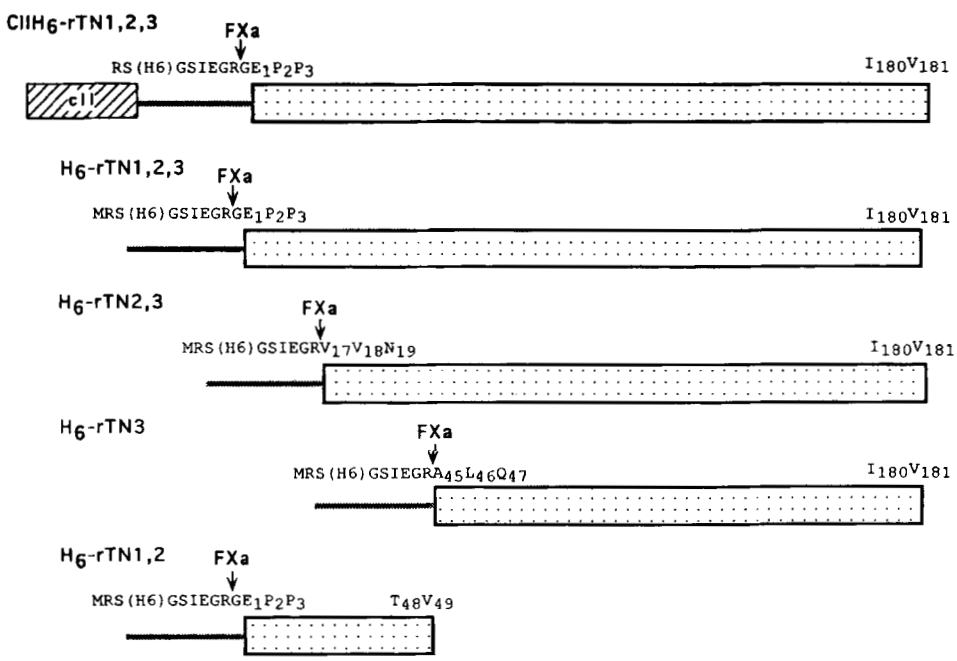

Fig. 1. Schematic representation of the recombinant tetranectin fusion proteins. The dotted boxes represents polypeptide sequences derived from TN exon 1, 2, and/or 3, respectively. The filled line represents the N-terminal fusion tail containing the six histidine residues and the FX_a cleavage site. The hatched box represents the 35-amino acid CII sequence in CIIH6-rTN123. Amino acid sequences of fusion tails and N/C terminal segments of each TN derived sequence are shown in one letter code above each construct.

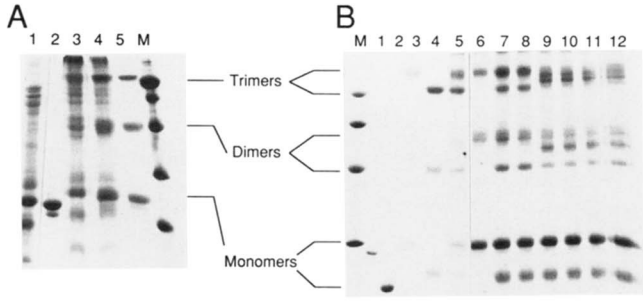

Fig. 2. Cross-linking analysis of human and recombinant tetranectin. **A:** SDS-PAGE (12%) cross-linking analysis of natural TN and rTN123. Human TN and rTN123 without DMSI, respectively (lanes 1 and 2). Human TN, 1:1 mixture of human TN and rTN123, and rTN123 cross-linked with DMSI after heating for two minutes, as described in Materials and methods (lanes 3, 4, and 5). Protein marker of 94, 68, 43, 30, and 20 kDa, top to bottom (lane M). **B:** Samples of rTN123, CIIH6-rTN123 and mixtures of both proteins were incubated with DMSI and analyzed by SDS-PAGE (12% gel). Before addition of DMSI, protein mixtures were subjected to subunit exchange by incubation at 70 °C for varying length of time. Protein marker of 94, 68, 43, and 30 kDa, top to bottom (lane M). CIIH6-rTN123 fusion protein (lane 1). r-TN123 (lane 2). DMSI treated CIIH6-rTN123, from two separate gels (lanes 3 and 6). DMSI-treated r-TN123 (lane 4). Identical samples of DMSI-treated mixtures of CIIH6-rTN123 and r-TN123 without heat exposure, on separate gels (lanes 5 and 7). Mixtures of r-TN123 and CIIH6-rTN123 heat treated for 2.5 s, 15 s, 2.5 min, and 10 min, respectively, before treatment with DMSI (lanes 8–11). 1:1 mixture of samples analyzed in lanes 8 and 10 (lane 12).

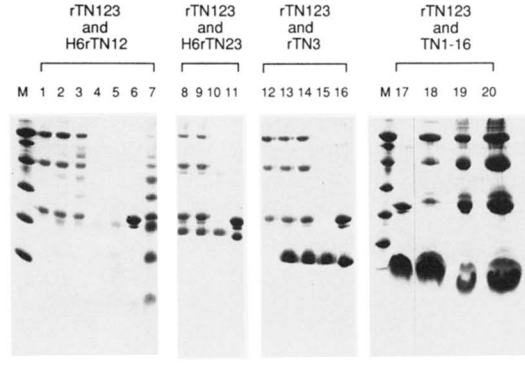

Fig. 3. Mapping of polypeptide segments in Tetranectin required for trimerization of tetranectin. The recombinant proteins rTN123, H6-rTN12, rTN23, and rTN3 and the synthetic oligopeptide TN1-16 or mixtures of rTN123 and each of the other were cross-linked by DMSI and analyzed by SDS-PAGE. Protein marker of 94, 68, 43, 30, 20, and 14.4 kDa, top to bottom (lanes M). rTN123 cross-linked with DMSI (lane 1). rTN123 and H6-rTN12 cross-linked with DMSI without and with heat treatment for two minutes, as described in Materials and methods (lanes 2 and 3). rTN12 cross-linked with DMSI (lanes 4 and 5). Mixture of rTN123 and H6-rTN12, no cross-linking (lane 6). Cross-linking of rTN123 and H6-rTN12 with H6-rTN12 in molar excess (lane 7). Cross-linking of rTN123 and rTN23 without and with heat treatment for two minutes (lanes 8 and 9). Cross-linking of rTN23 (lane 10). Mixture of rTN123 and rTN23 without cross-linking (lane 11). rTN123 cross-linked by DMSI (lane 12). Cross-linking of rTN123 and rTN3 without and with heat treatment for two minutes (lanes 13 and 14). Cross-linking of rTN3 (lane 15). Mixture of rTN123 and rTN3, no cross-linking (lane 16). Mixture of rTN123 and TN1-16, no cross-linking (lane 17). Mixtures of rTN123 and TN1-16 cross-linked with DMSI without and with heat treatment for two minutes (lanes 18 and 19). Sample as in lane 19 after 10 times concentration by precipitation with trichloroacetic acid (lane 20).

of rTN123. Likewise, the derivative rTN23, containing exon 2 and the CRD, appeared monomeric after cross-linking and was found not to interfere with trimerization of rTN123 during subunit exchange. Dimeric molecules found at low abundance in the sample probably reflects contaminating misfolded disulfide bridged dimers. The fusion protein H6-rTN12 and the synthetic peptide TN1-16 both formed homo-trimers upon cross-linking and generated hetero-trimes with rTN123 after subunit exchange. Because of the difference in size of full length TN (rTN123) and H6rTN12 the possible nine protein bands resulting from chemical cross-linking are: the four trimers $\{(rTN123)_3$, $(rTN123)_2(H6rTN12)$, $(rTN123)(H6rTN12)_2$, and $(H6rTN12)_3\}$ at approximately 95 kDa , 50 kDa, 37 kDa, and 20 kDa, respectively; the three dimers $\{(rTN123)_2$, $(rTN123)(H6rTN12)$, and $(H6rTN12)_2\}$ at approximately 45 kDa, 30 kDa, and 15 kDa, respectively; and the two monomers rTN123 at 23 kDa and H6rTN12 at 9 kDa.

High concentrations of the peptide TN1-16 were required to visualize cross-linking or interference with cross-linking of rTN123. Five of the 16 residues in TN1-16 are lysine. One likely reason for the requirement for high concentrations of the peptide TN1-16 to interfere with rTN123 trimerization is, therefore, that reaction of the five TN1-16 lysine residues with DMSI will significantly alter the structural properties of the peptide.

Taken together, the cross-linking analysis of the rTN derivatives shows that TN, like the collectin group of C-type lectins, is, in fact, a trimeric molecule and that amino acid residues directly shown to be involved in trimerization of the TN monomer are located in the N-terminal region of the protein (E1–D16).

Gelfiltration analysis of the rTN123 and rTN23 indicate that both proteins are trimers in solution (K_{av} values of 0.27 and 0.29, respectively), whereas rTN3 appeared monomeric (K_{av}: 0.41).

Discussion

Tetranectin was first described a decade ago (Clemmensen et al., 1986). Although biochemical and physiological studies of TN have progressed over the years to produce a basic description of amino acid and gene sequence, including characterization of possible interactions with other macromolecules and characterization of tissue localization in normal and pathological tissue, the precise physiological role of TN remains unresolved.

In terms of relation to known protein families, TN appears to meet the criteria for a C-type lectin. It binds complex polysaccharides in a Ca^{2+} dependent manner and its sequence encodes a single long-form CRD. One outstanding difference that has led to categorization of TN as belonging to a special class of lectins was the belief that TN is a tetrameric protein.

Our chemical cross-linking analysis of natural and recombinant TN has shown that the protein is a trimer, and that amino acid residues N-terminal to the CRD are required for trimerization of the monomeric subunits like in the collectin protein family of C-type lectins (Weis et al., 1991; Crouch et al., 1994; Ogasawara and Voelker 1995).

The rTN23 protein appears trimeric in solution, although it cannot be cross-linked, probably because it lacks appropriately positioned lysine residues in the trimerization domain. The amino acid sequence encoded by TN exon two is, like the neck regions of the collectins, characterized by the heptad repeats of aliphatic residues at a and d positions (Fig. 4). This sequence pattern is considered important for the formation of the observed triple-stranded α-helical coiled coil structure of the MBPs (Sheriff et al., 1994; Weis &

Fig. 4. Heptad repeat structure of tetranectin exon 2 and neck regions of mannose-binding proteins. Comparison of the amino acid sequence of TN exon 2 with the neck-regions of rat and human MBP. Aliphatic residues in the TN sequence corresponding to aliphatic residues in the heptad repeats of the MBPs, that form contact points in the triple-stranded α-helical coiled coil structure are shown in boldface.

Drickamer, 1994), and is indicative of a similar structural organization of the TN protein.

Notably, the rTN23 subunit is only poorly, if at all, capable of displacing the rTN123 subunit in the assembly of the trimer. This result, together with the observed ability of the peptide TN1-16 to interfere with TN123 trimerization, is interpreted as suggestive of a role of the exon one encoded sequence as stabilizing the trimeric organization of the molecule.

Materials and methods

cDNA cloning and production in E. coli of recombinant human Tetranectin and derivatives

Total RNA was isolated from human placenta as described by Chomczynski and Sacchi (1987). First-strand oligo-dT–primed cDNA was synthesized from 5 μg total human placental RNA using a standard procedure. The cDNA encoding the mature TN single-chain subunit (amino acid residues E1–V181, amino acid numbering according to Fuhlendorff et al., 1987) was amplified in a polymerase chain reaction (PCR), essentially according to (Saiki et al., 1988). One-tenth of the first-strand cDNA material was used as template. The primers used in the PCR were (the underlined nucleotides encode a FX_a cleavage site): TN-N: 5′-CCTGGATC CATCGAGGGTAGGGGCGAGCCACCAACCCAG-3′ and TN-C: 5′-CCGAAGCTTACACGATCCCGAACTG-3′. The N- and C-terminal oligonucleotide primer sequences were derived from the published genomic sequence (Berglund & Petersen, 1992). The amplified DNA fragment was cut with Bam HI and Hind III, isolated after agarose gel electrophoresis and subcloned into the *E. coli* expression vectors pT7H6 (Christensen et al., 1991) and pT7CIIH6, yielding pT7H6-TN123 and pT7CIIH6-TN123. Both vectors (pT7H6 and pT7CIIH6) express cloned sequences as fusion proteins. pT7CIIH6 was derived from pT7H6 by insertion of a DNA fragment excised with Nde I and BamH I from pLCII (Nagai & Thøgersen 1987) encoding the N-terminal 35 amino acid residues of the λCII protein in the 5′ end of the fusion tail. The plasmids pT7H6-TN12, pT7H6-TN23, and pT7H6-TN3 were constructed by cloning amplified DNA fragments containing exon 1 and 2 or exon 2 and 3, respectively into pT7H6. Plasmid pT7H6-TN123 was used as template in the PCR reactions together with the following oligonucleotide primers: TN-N, TN-C, TN-N17: 5′-GGCGGATCCATCCAGGGTAGGGTTGTGAACACAAA GATG-3′, TN-N66: 5′-CCTGGATCCATCGAGGGTAGGGCCCT GCAGACGGTC-3′, and TN-C70: 5′-CGGAAGCTTAGACCGTC TGCAGGGC-3′. The nucleotide sequence of the expression plasmid inserts were verified using the Sequenase ver. 2.0 DNA sequencing kit (USB Corp. USA). A schematic overview of the expressed fusion proteins is given in Figure 1.

Expression in E. coli of recombinant Tetranectin fusion proteins

The recombinant human TN monomer fusion proteins H6-rTN12, H6-rTN23, H6-rTN3, H6-rTN123, and CIIH6-rTN123 were produced by expressing the corresponding plasmids in *E. coli* BL21 cells, as described (Studier et al., 1990). Exponentially growing cultures at 37 °C were at OD_{600} 0.8 infected with bacteriophage λCE6 at a multiplicity of approximately 5. Cultures were incubated further for three hours at 37 °C before harvesting cells by centrifugation. Cells were resuspended in 150 mL of 0.5 M NaCl, 10 mM Tris-HCl pH 8, and 1 mM EDTA. Phenol (adjusted to pH 8) was added and the mixture sonicated to extract the total protein. Protein was precipitated from the phenol phase by addition of 2.5 volumes of ethanol and harvested by centrifugation. The protein pellet was dissolved in a buffer containing 6 M guanidinium chloride, 50 mM Tris-HCl pH 8, and 0.1 M dithioerythriol. Following gel filtration on Sephadex G-25 (Pharmacia, Sweden) into 8 M Urea, 1 M NaCl, 50 mM Tris-HCl pH 8, and 10 mM 2-mercaptoethanol, the crude protein preparation was applied to a 40 ml Ni^{2+} loaded nitrilo-triacetic-acid agarose (NTA-agarose) column. The Ni^{2+} NTA-agarose column was then washed with Buffer I (200 mL of 8 M urea, 1 M NaCl, 50 mM Tris-HCl pH 8, and 10 mM 2-mercaptoethanol) and Buffer II (100 mL 6 M guanidinium chloride, 50 mM Tris-HCl pH 8, and 10 mM 2-mercaptoethanol). Fusion protein was eluted with Buffer II containing 10 mM EDTA pH 8 and the eluate was gel filtered on Sephadex G25 into Buffer I.

Refolding and processing of the Tetranectin fusion proteins

Purified reduced and denatured fusion proteins H6-rTN3, H6-rTN23, H6- rTN123, and CIIH6-rTN123 were refolded using a new cyclic procedure (TLH, ME, and HCT int. pat. appl. WO94/18227, and in prep.). Fusion proteins in Buffer I were mixed with 100 mL Ni^{2+} NTA-agarose. The resin containing bound protein was then packed into a 5-cm diameter column, washed with Buffer I supplemented with $CaCl_2$ to 2 mM, and subjected to the cyclic refolding procedure. After completion of cycling the H6-rTN3, H6-rTN23, H6-rTN123, or CIIH6-rTN123 fusion proteins were eluted from the Ni^{2+} NTA-agarose column with a buffer III containing 0.5 M NaCl, 50 mM Tris-HCl pH 8, and 25 mM EDTA.

The fusion protein H6-rTN12 was applied to the Ni^{2+} NTA-agarose matrix in buffer I and refolded by removal of the denaturant in a linear gradient over five column volumes before elution of H_6r-TN12 in Buffer III. The fusion proteins H_6-rTN123, H_6-rTN23 and H_6-rTN3 were cleaved with FX_a at 4 °C overnight at a molar ratio of 1:300. After FX_a cleavage, the recombinant proteins were concentrated by ultrafiltration on YM10 or YM30 membranes (Amicon, USA). After 10-fold dilution of the protein sample with 2 mM $CaCl_2$ the recombinant proteins rTN123, rTN23,

and rTN3 were isolated by ion-exchange chromatography on Q-Sepharose (Pharmacia, Sweden) in linear gradients (0–0.5 M NaCl) over 10 column volumes in a buffer containing 10 mM Tris-HCl pH 8 and 2 mM $CaCl_2$.

Chemical cross-linking of tetranectin and derivatives

TN, isolated from human plasma essentially as described by Clemmensen et al. (1896), the refolded recombinant TN derivatives rTN123, rTN3, rTN23, together with the fusion proteins CIIH6-rTN123 and H6-rTN12, and a synthetic peptide (TN1–16), amino acid sequence EPPTQKPKKIVNAKKD (Schafer-N, Copenhagen, Denmark) or mixtures of these derivatives at 1 mg/mL concentrations in cross-linking buffer (0.1 M sodium borate, pH 9.1), were incubated with DMSI. Aliquots (10 μL) of protein solution were incubated with 1 μL aliquots of DMSI stock solution (20 mg/mL in cross-linking buffer) for 30 min at 25 °C before addition of 2 μL quenching buffer (3 M Tris-HCl buffer, pH 9). Subunit exchange between pre-formed homo-oligomers was induced by subjecting protein mixtures to heat shock treatment. Aliquots (5 μL) of each protein solution (1 mg/mL stocks) were mixed at 0 °C in standard polypropylene microcentrifuge tubes, transferred to a water bath at 70 °C for the time spans indicated, and then further incubated for 15 min at 25 °C in a water bath before reaction with DMSI.

Prior to analysis by SDS-PAGE (12% gels) of the cross-linked products the reaction samples were boiled in the presence of SDS and mercaptoethanol.

Analytical gelfiltration analysis of rTN proteins

Analytical gelfiltration was performed on a Superose 12 HR 10/30 column (Pharmacia, Sweden) with a total volume of 25 mL in 100 mM NaCl and 50 mM Tris-HCl pH8 and a flow rate of 0.2 mL/min. The K_{av} value is defined by $K_{av} = (Ve - Vo)/(Vc - Vo)$.

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