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REDUCTIVE CLEAVAGE OF THE DISULFIDE BONDS OF THE COLLAGEN IV NONCOLLAGENOUS DOMAIN IN AQUEOUS SODIUM DODECYL SULFATE: ABSENCE OF INTERMOLECULAR NONDISULFIDE CROSS-LINKS

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The subunits of the collagen IV hexameric, noncollagenous NC1 domain obtained from bovine aorta, glomerular basement membrane, alveolar basement membrane and placental basement membrane are predominantly dimers. A large fraction of the dimers had been thought to be linked by nondisulfide bonds because they were resistant to cleavage by 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, at 100°C. However, if an unusually high concentration of 2-mercaptoethanol, e.g., 40% (v/v), is used, complete conversion of dimers into monomers is achieved, indicating the lack of intersubunit nondisulfide cross-links. Electrophoresis patterns indicate that some of the intermolecular disulfide bonds of the dimers are more resistant to reduction in aqueous SDS than are some of the intramolecular disulfide bonds.

Collagen IV, the major component of basement membranes, in obtaining its supramolecular structure, self-associates by forming a tetramer (of triple-helical molecules) at its amino-terminal end and a dimer at the carboxyl-terminal end (1). The globular noncollagenous NC1 domain at the carboxyl-terminal end of collagen IV is an important structural element because it is involved in dimerization (2) and, furthermore, contains the epitope that reacts with autoantibodies from patients with Goodpasture syndrome (3). The NC1 domain is released as a hexamer, composed of monomeric and dimeric subunits, by digestion of collagen IV with bacterial collagenase.

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The dimeric NC1 subunits are formed by covalent cross-linking of monomeric subunits of different collagen IV molecules that have dimerized. In a major paper on the NC1 domain of collagen IV from a mouse tumor, it was proposed that both disulfide and non-disulfide cross-links are involved in dimerization (2). The basis for the proposal was that a fraction of the dimers were not dissociated by treatment with 5% 2-mercaptoethanol (v/v) in the potent denaturant, 2% SDS. Additional support for the presence of non-disulfide cross-links came from similar results on NC1 domains from collagen IV of bovine glomerular membrane lens basement membrane and placental basement membrane (4); alveolar basement membrane (5); and bovine aorta (6). In the study described here, we re-investigated the reductive cleavage of NC1 dimers from several sources and found that the dimers can be converted essentially completely into monomers, but only if unusually high concentrations of 2-mercaptoethanol are used.

MATERIALS AND METHODS

Isolation of NC1 hexamer. NC1 hexamers of placental-, alveolar- and glomerular basement membrane collagen were prepared as described previously (4,5,7). To prepare the NC1 domain of aorta collagen, adult bovine aorta (Pel-Freez Biologicals, Rogers, AR) was cut open longitudinally with scissors, the adventitia was stripped off and discarded. The remaining material was ground and then extracted at 4°C for 24 h in of 0.5 M KCl, 0.1 M Tris-HCl, pH 7.5, 1 mM phenylmethanesulfonyl fluoride, 10 mM EDTA, 25 mM 6-aminohexanoic acid, 4 mM N-ethylmaleimide, 5 mM benzamidine-HCl (7-10 ml/g tissue). Then, the ground tissue was suspended in 6 M guanidine-HCl, 50 mM Tris-HCl, pH 7.5 (6 ml/g tissue) and stirred gently at room temperature for 36 h. Then, the same procedure used for glomerular basement membrane (7) was used to prepare aorta collagen IV NC1. Calf skin collagen was obtained from Sigma Chemical Co.

Reduction and carboxymethylation of aorta collagen IV NC1 domain dimer. The aorta NC1 dimer was prepared from the hexamer using a published method (8). To reduce disulfide bonds, the dimer was heated in 6 M guanidine-HCl, 1.3% 2-mercaptoethanol, 1.4 M Tris-HCl, pH 7.5, at 100° C for 10 min. Then the sample was cooled to room temperature and the protein sulfhydryl groups were reacted with iodoacetate according to the procedure of Crestfield et al. (9).

Electrophoresis techniques. SDS-polyacrylamide gel electrophoresis was carried out with 4-22% linear gradient gels and the discontinuous buffer system of Laemmli (10). To reduce NC1 disulfide bonds, the protein was heated for 5 min at 100°C in sample buffer containing 2-mercaptoethanol or dithiothreitol, and 2% SDS; several concentrations of 2-mercaptoethanol (5% to 40% v/v) or dithiothreitol (5% to 60% w/v) were used. The gels were stained with Coomassie brilliant blue R 250.

Analysis of lysine-derived collagen cross-links. The presence of collagen cross-links in the aorta type IV collagen NC1 domain was investigated by ion-exchange and HPLC systems. The ion-exchange system involved the use of a CF1 pre-column to concentrate the cross-links and then separation on an LKB 4400 Autoanalyzer employing a modified sodium citrate gradient as recently described in detail (11). The NC1 samples were hydrolyzed before and after reduction with sodium borohydride prior to analysis. The cross-links eluted from the CF1 column were also analyzed on an LKB HPLC system using a 15 x 0.46 cm column in the reversed-phase mode. Buffer A (water) and buffer B (tetrahydrofuran, 0.5% trifluoroacetic acid) were used to make a gradient from 0-11% tetrahydrofuran over a 45 min period.

RESULTS AND DISCUSSION

Samples prepared as described above were analyzed by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions; the results are shown in Figures 1-4. The effect of various 2-mercaptoethanol concentrations on the electrophoretic mobility of the subunits of the aorta (A) and glomerular basement membrane (G) NC1 domain hexamer is illustrated in Figure 1. In the absence of 2-mercaptoethanol (the pair of lanes under "0"), a set of dimer bands was found, with apparent M_r between 28,000 and

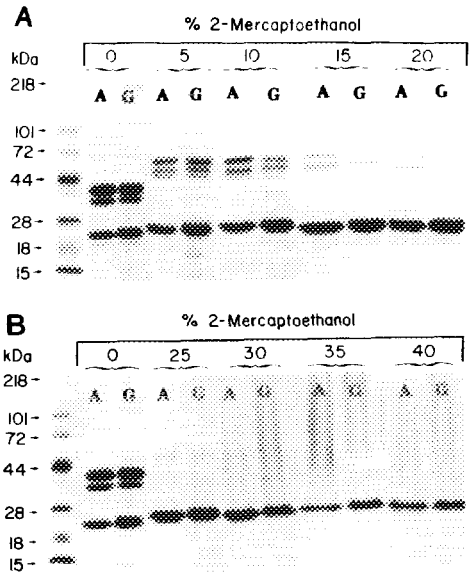

Figure 1. A) and B) **The effect of 2-mercaptoethanol concentration on the reduction of intermolecular disulfide cross-links in NC1 domain dimers from aorta and glomerular basement membrane collagen IV.** SDS-polyacrylamide gel electrophoresis was carried out as described in **METHODS**. Lane 1 is a set of standards with the indicated molecular masses. A is aorta NC1 domain and G is glomerular basement membrane NC1 domain. The 2-mercaptoethanol concentration (v/v) is indicated above each pair of lanes.

44,000, and a set of monomer bands with $\underline{M}_r$ between 18,000 and 28,000. After reduction in 2% SDS containing 5% 2-mercaptoethanol, some of the dimer fraction was converted into monomer. In addition, the apparent $\underline{M}_r$ of the remaining dimers increased significantly (to values ≥ 44,000). The increase in apparent $\underline{M}_r$ of the dimers indicates that some (or all) of the intramolecular disulfide bonds were reduced, resulting in a decreased mobility because of an increase in hydrodynamic volume due to loss of disulfide loops and perhaps concomitant loss of residual hydrophobic interactions between sidechains. Those intramolecular disulfide bonds were more susceptible to reduction in 5% 2-mercaptoethanol than some of the intermolecular disulfide bonds. The resistance of the intermolecular disulfide bonds to reduction could be overcome by using higher concentrations of 2-mercaptoethanol, with essentially complete conversion of dimers into monomers obtained at 35% 2-mercaptoethanol. The results obtained for glomerular basement membrane- and aorta NC1 were also found for alveolar basement membrane NC1 and placental basement membrane NC1 (Fig. 2). In both cases, all of the dimer fraction could be converted into monomer with 40% 2-mercaptoethanol. The resistance of NC1 dimer intermolecular disulfide bonds to reduction was also found when dithiothreitol was used as the reductant for aorta NC1 instead of 2-mercaptoethanol (Fig. 3). To test the effect of the type of denaturant on reduction of intermolecular disulfide bonds, we used 6 M guanidine-HCl and, only, 1.3% 2-

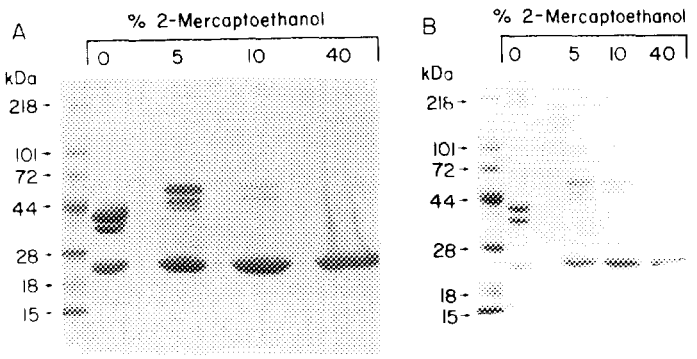

Figure 2. **The effect of 2-mercaptoethanol concentration on the reduction of intermolecular disulfide cross-links in NC1 domain dimers from alveolar- and placental basement membrane type IV collagen.** SDS-polyacrylamide gel electrophoresis was carried out as described in **METHODS**. Lane 1 in each panel is a set of standards with the indicated molecular masses. The results for alveolar basement membrane NC1 are shown in panel A and those for placental basement membrane NC1 are shown in panel B. The 2-mercaptoethanol concentration (v/v) is indicated above each lane.

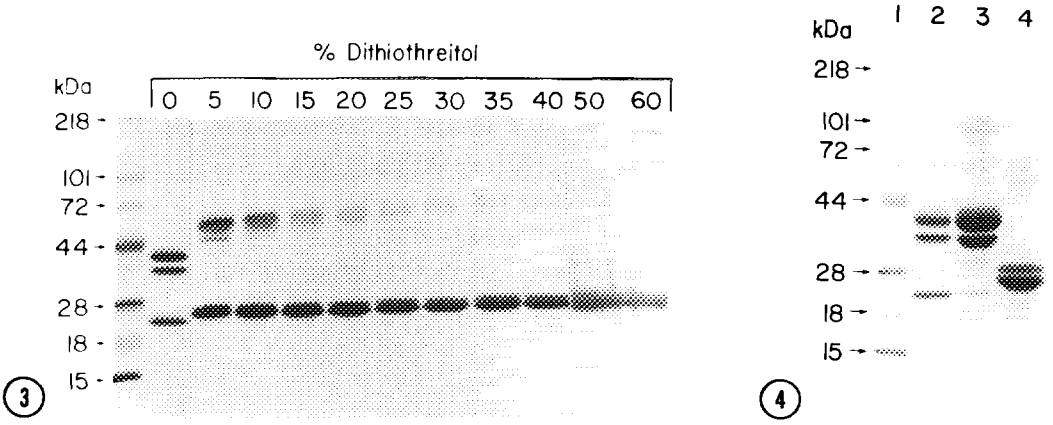

Figure 3. **The effect of dithiothreitol concentration on the reduction of intermolecular disulfide cross-links in NC1 domain dimers from aorta type IV collagen.** SDS-polyacrylamide gel electrophoresis was carried out as described in **METHODS**. Lane 1 is a set of standards with the indicated molecular masses. The dithiothreitol concentration (w/v) is indicated above each lane.

Figure 4. **The conversion of aorta NC1 dimer into monomer by reduction of disulfides and subsequent alkylation.** SDS-polyacrylamide gel electrophoresis was carried out as described in **METHODS**; no reductant was used. Lane 1 is a set of standards with the indicated molecular masses, lane 2 is original NC1 hexamer, lane 3 is NC1 dimer, and lane 4 is the monomeric product obtained by denaturation and reduction of the dimer, followed by alkylating the resulting cysteine sidechains with iodoacetate.

mercaptoethanol at 100° C. After reduction and alkylation of aorta NC1 dimer, SDS-polyacrylamide gel electrophoresis in the absence of a reductant revealed the complete absence of dimers (Fig. 4, lane 2).

With respect to non-disulfide cross-links, analysis of the aorta NC1 domain hexamer by ion-exchange chromatography and reversed-phase HPLC failed to reveal any of the known collagen cross-links. Although the 7 S region of collagen IV contains the lysine-derived cross-links (12) these results clearly indicate their absence from the NC1 domain.

Our results, taken together, show that some (or none) of the intermolecular disulfide bonds of NC1 dimers are, to some extent, protected from reduction in the SDS dimer complex or, alternatively, re-form after cooling in 2% SDS, 5% 2-mercaptoethanol. This unusual circumstance led to an erroneous suggestion that there are non-disulfide intermolecular cross-links in NC1 dimers from several tissues (4-6). The results of the present study are likely to be useful in future studies on the Goodpasture epitope, which is located in the α3(IV)NC1 domain and is involved in the Goodpasture syndrome, an autoimmune disease that

affects kidney and lung (3). The affinity of the epitope for autoantibody is decreased when disulfide bonds are reduced (13). The selective resistance of some of the disulfide bonds to reduction in 2% SDS may provide a means to determine which of them are required for integrity of the epitope.

ACKNOWLEDGMENTS

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