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Chemical crosslinking with disuccinimidyl tartrate defines the relative positions of the two antiparallel coiled coils of the desmin protofilament unit

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Filaments formed by desmin, the myogenic intermediate filament protein, were crosslinked with the lysine specific crosslinker DST (disuccinimidyl tartrate; 0.64 nm span) and three DST crosslinked peptides were characterized. Two correspond to crosslinks previously obtained with the longer crosslinker EGS (ethylene glycol bis(succinimidylsuccinate), 1.61 nm span) which defined the antiparallel on-stagger relationship of neighbouring coiled coils. The two DST crosslinks now provide the relative positions of the coiled coils within a limit of about 9 α-helical residues. The third DST crosslink most likely connecting two helices of a single coiled coil gives a direct measure of the distance spanned in DST crosslinks.

Desmin; Coiled coil; Crosslinking; Tetramer; Disuccinimidyl tartrate

1. INTRODUCTION

The structural proteins of cytoplasmic intermediate filaments (IF) of vertebrates have a clearly defined three-domain structure. A central α-helical rod domain of some 310 residues is flanked by non-helical terminal domains, which vary greatly in sequence and length [1,2]. Two rod domains form a double stranded, parallel coiled coil and two coiled coils align into the relatively stable tetrameric protofilaments. These polymerize in a number of still poorly understood steps into the filament. Although the rods form the framework of IF, the amino-terminal head domains contribute to filament formation and stability. The proteolytically excised rod domain remains a tetramer even under polymerisation conditions [3,4] (for reviews see [1,2,5,6]).

The alignment of the two coiled coils in the tetramer has long been a matter of debate. More recent results obtained from paracrystals [7], hydrodynamic data [8], biochemical and electronmicroscopical observations [9,10] as well as from chemical crosslinking data [11] all favour a similar model. Here the two coiled coils are aligned in an antiparallel arrangement using a stagger, which corresponds to approximately 30–40% of the length of a single coiled coil. The orientation of the two coiled coils is such that the amino-terminal ends (helices 1A, 1B and 2A) form the inner region of the tetramer while the carboxytermini (helices 2B) extend at opposite ends.

In the crosslinking study of desmin filaments and rods two crosslinks formed between neighbouring coiled coils by the lysine specific crosslinkers EGS have been characterized [11]. They link two lysine residues of helix 1A of the first coiled coil to two lysine residues in helix 2A of the second coiled coil. Although these crosslinks define the antiparallel tetramer they could not describe the alignment of the coiled coils with an accuracy better than a length corresponding to about 15–20 residues in α-helical conformation. The reasons for this relatively long distance lie in the span width of the crosslinker (1.61 nm for EGS) and the unknown orientation of the crosslinks versus the axis of the tetramer. Thus it is not known whether the crosslinks are parallel or perpendicular to the long axis of the tetramer or occupy an intermediate position. Here I have refined the model using the shorter crosslinker DST (0.64 nm). I have isolated two DST crosslinks from desmin filaments, which reflect the same lysine residues previously connected by EGS. Although it seems puzzling at first that identical lysine residues can be bridged by crosslinkers with such different span widths as EGS and DST, the specific geometry of the two aligned coiled coils offers a simple explanation. The DST crosslinks obtained provide the relative positions of the neighbouring coiled coils within a limit of about 9 α-helical residues.

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Abbreviations: DST, disuccinimidyl tartrate; EGS, ethylene glycol bis(succinimidylsuccinate); IF, intermediate filament; TFA, trifluoroacetic acid.

2. MATERIALS AND METHODS

2.1. *Crosslinking*

Desmin, purified from chicken gizzard [12], was polymerized into filaments by dialysis against crosslinking buffer. This was 0.1 M tri-

ethanolamine/HCl at pH 8.0 supplemented with 0.17 M NaCl and 1 mM 2-mercaptoethanol. The protein concentration was adjusted to approximately 1 mg/ml. DST (Pierce, Rockford, IL, USA) was dissolved in dimethylsulfoxide at 30 mg/ml and diluted into the protein solution to a final concentration of 1 mg/ml. Crosslinking proceeded at 37°C for 30 min. The reaction was stopped by the addition of an ethanolamine/HCl stock solution at pH 8.0 to a final concentration of 0.1 M followed by an incubation for 15 min at 37°C. The DST crosslinked material was recovered by dialysis against water and lyophilization. It was treated with CNBr in 70% formic acid. Resulting fragments were separated by gel filtration on a Sephadex G100 column in 8 M urea containing buffer (0.1 M Tris-HCl, pH 7.5, 1 M 2-mercaptoethanol), as described [11]. Elution of fragments was monitored by SDS-PAGE. Fractions of interest were dialyzed against water and lyophilized. Aliquots of the crosslinked fragments were digested with trypsin and the resulting peptide mixture was separated by gel filtration using Sephadex G75 sf equilibrated in 50 mM triethanolamine/HCl buffer at pH 8.0. Peptide containing fractions were separated on C_{18} reversed phase HPLC columns run in 0.1% TFA. Eluted peptides or mixtures of peptides were identified by a partial sequence analysis. Peptides of interest were either further purified by HPLC in phosphate buffer at pH 6.0 or used directly for complete sequence determination. Mass determination was done by plasma desorption time of flight mass spectrometry. Automated Edman degradation was performed with a Model 810 Knauer protein sequencer (Berlin, Germany). Modified lysine residues were identified along the procedures outlined before [11].

3. RESULTS

Crosslinking of desmin filaments with DST and identification of the crosslinked lysine residues followed the procedure previously established for EGS crosslinking [11]. Crosslinking with DST did not alter the electron microscopical appearance of the filaments (data not shown) as already documented for EGS crosslinked filaments [11]. Chromatography of CNBr fragments on Sephadex G100 in 8 M urea yielded a profile similar to that obtained with EGS treated filaments. Most of the material eluted close to the exclusion volume of the column. In SDS-PAGE it provided a smear close to the upper end of the gel. Sequence analysis of the material revealed the presence of the large fragment representing the entire helix 1B (residues 146–254), the fragment covering most of helix 1A (residues 89–127) and the fragment following helix 1B on the carboxy-terminal side. This covers helix 2A, the following linker L2 and the amino-terminal part of helix 2B (residues 255–352). Additional fragments present were some smaller peptides from helix 2B, and uncleaved overlap peptides, which are of no interest in this study. A digest of the material with trypsin was subjected to gel filtration on Sephadex G75 sf and the resulting elution profile was screened by sequence analysis for potential cross-linked peptides. Candidate fractions, which eluted early from the column, were subjected to one or two C_{18} reversed phase HPLC separations (Fig. 1), which were performed as described for EGS crosslinks [11]. Three peptides containing DST crosslinks were finally isolated and characterized. DST1 contained the sequences of residues 110–118 and 255–266 in equal molar amounts, due to the crosslink between lysine residues 116 and 258 (Fig. 1E). DST2 had equal molar amounts of residues 97–109 and residues 270–288 (Fig. 1E). The crosslink of DST2 must link lysine residues 100 and 278 since Lys-288 was accessible to tryptic cleavage. That Lys-288 is indeed not modified was directly documented by the sequence analysis. Thus except for the crosslinker moiety, DST1 and DST2 are identical with the crosslinked peptides EGS1 and EGS2 (Fig. 2) found after crosslinking with EGS [11]. Mass spectrograms essentially confirmed the assignments for DST1 and DST2 (Fig. 1E). However the measured mass values were consistently by 18 Da lower than the mass calculated from the two linked peptides and the crosslinker moiety. The reason for this small discrepancy is not fully understood since a similar difference was not observed in the EGS crosslinks [11]. However, the loss of one water molecule from the vicinal hydroxyl groups in the DST crosslinker moiety seems a likely explanation. Such β elimination would be favoured by the slightly alkaline conditions used during the crosslinking reaction and the subsequent dialysis step (see section 2).

The third crosslinked peptide DST3 contained residues 172–182 and residues 181–184 in equal molar amounts. The DST crosslink connects lysine 180 of the longer peptide with Lys-182 of the tetrapeptide (Fig. 1E). Lys-182 of the longer peptide is unmodified as shown by sequence analysis and the fact that it occupies a carboxy-terminal position after tryptic cleavage. The tetrapeptide arose by cleavage at an unmodified Lys-180 and Arg-184. Mass spectroscopy confirmed the assignment but again showed a value, which was by 18 Da lower than the calculated mass (see above).

4. DISCUSSION

We have previously crosslinked rods, protofilaments and full desmin filaments with the lysine-specific crosslinker EGS (1.61 nm span width) and characterized two crosslinks formed between neighbouring coiled coils of the tetramer. EGS1 and EGS2 connect two lysine residues (positions 100 and 116) of helix 1A from the first coiled coil with two lysine residues (positions 278 and 258) of helix 2A of the second coiled coil in reversed order, i.e. Lys-100 is linked to Lys-278 and Lys-116 is connected to Lys-258 (see Fig. 2). These results presented strong support for the antiparallel and staggered alignment of the two coiled coils. The relative long span width of EGS (1.61 nm) restricted the possible alignments within borders, which are about 20 α helical residues apart [11]. We have now shown (Fig. 1) that the much shorter DST molecule (span width 0.64 nm) used on desmin filaments provides the same two crosslinks, except for the moiety of the reagent, as earlier documented with EGS1 and EGS2. This finding improves the accuracy for the alignment of the two coiled coils considerably as the borders are now narrowed to within

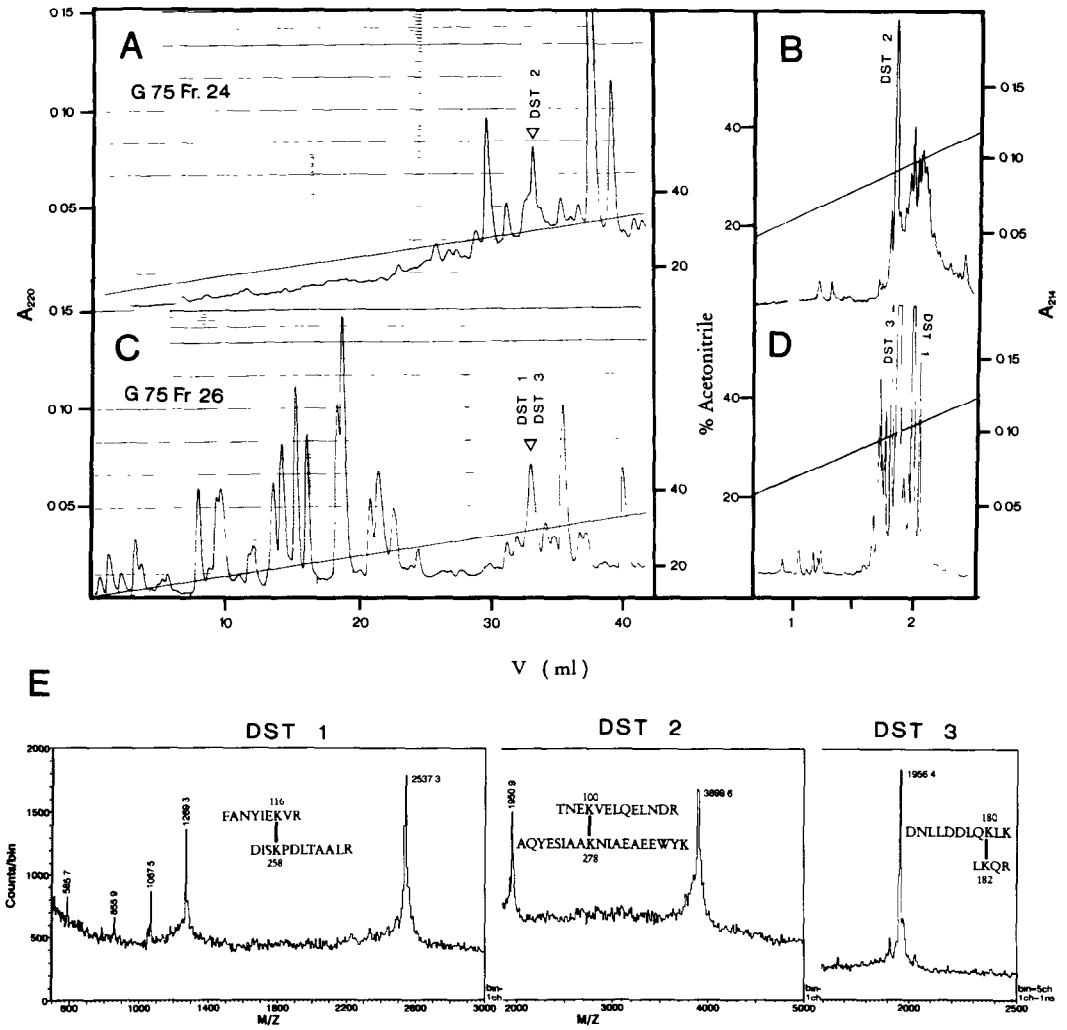

Fig. 1. Purification and characterization of DST-crosslinked tryptic peptides. Crosslinked desmin filaments were treated with CNBr. Gel filtration on S100 provided high molecular weight material, which was digested with trypsin and chromatographed on Sephadex G75 sf. A and C show the elution profiles of two G75 sf fractions in HPLC using 0.1% TFA. Both fractions (24 and 26) were from the leading edge of the main peak eluted from G75 (this profile was very similar to that given in Fig. 4A in [11]. B and D show the final purification of the crosslinked peptides by micro HPLC at pH 6.0. The peaks marked DST1, 2 and 3 in A and C were recovered in pure form in B and D. Peaks DST1, 2 and 3 from B and D show the sequences and mass spectrograms given in E. The numbers at the crosslinked lysine residues shown in each spectrograph give the position in the full sequence of chicken desmin [3] Crosslinked lysines (K) are connected by a vertical line.

$2 \times 0.64 = 1.28$ nm or 9 residues in helical conformation. Although it seems puzzling at first that identical lysine pairs can be bridged by crosslinkers differing in span width as strongly as 1.61 nm (EGS) and 0.64 nm (DST) the special structural characteristics of the tetramer offer a ready explanation. In a double-stranded coiled coil each particular residue is present twice and occupies symmetrical positions. Two aligned coiled coils present the same pair of lysines twice and, with high probability, at different distances (Fig. 2B). Thus, efficient inter coiled coil crosslinking can be obtained by a shorter as well as by a longer crosslinker. For obvious reasons the shorter crosslinker defines the actual alignment with higher accuracy as the longer one. Fig. 2A displays therefore the previously proposed arrangement of the two coiled coils [11] in a slightly modified form due to the shortened distances now obtained for DST1 and DST2. Although a detailed calculation of the relative yield of the DST crosslinked peptides was not performed (for EGS see [11]) all three peptides were obtained at approximately 30%. This is a high yield considering that crosslinks in coiled coil structures are probably maximally formed at 50% yield [11]. The crosslink DST3 offers a possible experimental measure for the distance bridged by DST. It connects lysine residues 180 and 182, which are located slightly prior to the middle of helix 1B (residues 146–241). DST3 most likely arises as a crosslink between the 1B helices of a single coiled coil although a link between neighbouring coiled coils of the tetramer or even between tetramers is not

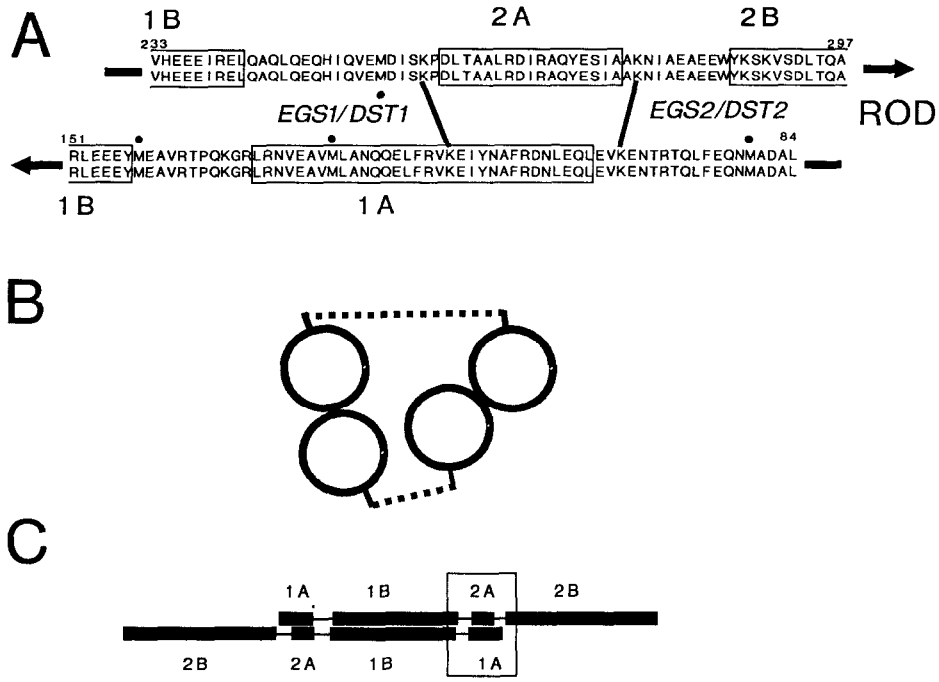

Fig. 2. Antiparallel on-stagger alignment of the two coiled coils in the rod tetramer. (A) The relevant part of the rod tetramer is presented by the sequence from the carboxy-terminus of helix 1B (H1B; all helical regions are boxed) into the amino-terminal end of helix 2B (H2B) of the first coiled coil (doubled sequences, upper row) and below by the sequences from helix 1A (H1A) into the amino-terminal end of helix 1B (H1B) from the second coiled coil [11] To account for the antiparallelity, sequences of the second coiled coil are written from right to left (see also direction of the thick arrows). The cross-links are marked by bars. The inter coiled coil cross-links DST1 and DST2 identified (see Fig. 1) connect lysine residues 100 and 278 (DST2) and 116 and 258 (DST1), respectively. The same crosslinks were previously found as EGS2 and EGS1, respectively Note that the crosslinkers EGS (1.61 nm) and DST (0 64 nm) differ strongly in span width. Dots point to cleavage sites for CNBr. (B) Example for a possible alignment of two double-stranded coiled coils (shown in cross-section), which would allow for the formation of the same crosslinks (dashed lines) based on crosslinkers with different span width (C) Summary of the proposed structure for the tetrameric rod. The two coiled coils (indicated by thicker bars) are aligned in an antiparallel arrangement in which the inner overlapping part is formed by the amino-terminal helices 1A, 1B and 2A. The area marked by the box is shown enlarged in (A).

yet fully excluded. The last possibility seems unlikely since such crosslinks in filaments are expected to form only at very low yields. If DST3 really reflects a crosslink between neighbouring helices of the same coiled coil the DST moiety spans the axial distance between lysine 180 and 182, i.e. 2 residues or 0.3 nm. This would argue for a more or less perpendicular orientation of the crosslinking moiety versus the axis of the coiled coil and fit the perpendicular distances of the two lysines (heptade positions c and e) in a coiled coil.

In a recent crosslinking study using epidermal keratins and oxidative bridging of cysteine residues [13] two additional types of tetrameric complexes have been defined: first, antiparallel and in-register, second, antiparallel and staggered, with helices 2B overlapping. The type of tetramer described in this study (antiparallel, helices 1A to 2A overlapping) was not detectable since appropriately placed cysteine residues are not present in the keratins used. Together, the three types of tetramers now defined allow the definition of the principal framework of intermediate filaments and support earlier suggestions based on more indirect evidence (e.g. [10,13–15]). Refinement of the alignment modes as obtained above is now necessary to obtain more detailed models.

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