

The Coiled Coil of In Vitro Assembled Keratin Filaments Is A Heterodimer of Type I and II Keratins: Use of Site-specific Mutagenesis and Recombinant Protein Expression

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Abstract. Recombinant DNA technology has been used to analyze the first step in keratin intermediate filament (IF) assembly; i.e., the formation of the double stranded coiled coil. Keratins 8 and 18, lacking cysteine, were subjected to site specific in vitro mutagenesis to change one amino acid in the same relative position of the α -helical rod domain of both keratins to a cysteine. The mutations lie at position -36 of the rod in a "d" position of the heptad repeat pattern, and thus air oxidation can introduce a zero-length cystine cross-link. Mutant keratins 8 and 18 purified separately from *Escherichia coli* readily formed cystine homodimers in 2 M guanidine-HCl, and could be separated from the monomers by gel filtration. Heterodimers with a cystine cross-link were obtained when filaments formed by the two reduced monomers were allowed to oxidize. Subsequent ion exchange chromatography in 8.5 M urea showed that only a single dimer species had formed. Diagonal electrophoresis and reverse phase HPLC identified the

dimer as the cystine containing heterodimer. This heterodimer readily assembled again into IF indistinguishable from those obtained from the nonmutant counterparts or from authentic keratins. In contrast, the mixture of cystine-stabilized homodimers formed only large aberrant aggregates. However, when a reducing agent was added, filaments formed again and yielded the heterodimer after oxidation. Thus, the obligatory heteropolymer step in keratin IF assembly seems to occur preferentially at the dimer level and not during tetramer formation.

Our results also suggest that keratin I and II homodimers, once formed, are at least in 2 M guanidine-HCl a metastable species as their mixtures convert spontaneously into heterodimers unless the homodimers are stabilized by the cystine cross-link. This previously unexpected property of homodimers explains major discrepancies in the literature on the keratin dimer.

THE basic subunit structure of all cytoplasmic intermediate filament (IF)¹ proteins of vertebrates comprises a globular head domain, a central α -helical rod domain of ~ 310 amino acids, and a non- α -helical tail domain (Geisler and Weber, 1982; Geisler et al., 1982; Steinert et al., 1983, 1985; Hanukoglu and Fuchs, 1983; Weber and Geisler, 1984). The rod domain shows over most of its length the heptad repeat pattern with hydrophobic residues in positions *a* and *d*, which is characteristic for α -helical coiled coil molecules (Crick, 1953; McLachlan and Stewart, 1975). The polypeptide chains of the double-stranded coiled coil are parallel and in register (Geisler and Weber, 1982; Weber and Geisler, 1984; Woods and Gruen, 1981; Gruen and Woods, 1983; Woods and Inglis, 1984; Parry et al., 1985; Quinlan et al., 1986).

Studies on soluble precursors of in vivo, as well as in vitro,

assembled IF revealed that the building unit is a tetramer comprising two coiled coils (Ahmadi and Speakman, 1978; Geisler and Weber, 1982; Quinlan et al., 1984, 1985; Ip et al., 1985; Parry et al., 1985; Soellner et al., 1985). Although contradictory results have been reported, recent experiments suggest that in the tetramer the coiled coils are oriented in an antiparallel manner (Geisler et al., 1985; Parry et al., 1985) and are staggered (Fraser et al., 1985; Potschka, 1986; Stewart et al., 1989). In paracrystals of the GFAP rod the dimers are antiparallel with the N termini overlapping by ~ 34 nm (Stewart et al., 1989).

Although all IF proteins seem to share this subunit structure, keratins differ from other IF proteins in several aspects. (a) They represent a complex multigene family of close to 30 different polypeptides, which are expressed in a tissue specific, developmentally regulated pattern (Moll et al., 1982; Fuchs et al., 1984). (b) They are clearly divided into two subclasses, types I and II keratins, which share only 30% sequence homology (Fuchs et al., 1981; Hanukoglu

1. *Abbreviations used in this paper:* DTE, dithioerythritol; IF, intermediate filament.

and Fuchs, 1983; Schiller et al., 1982; Tseng et al., 1982). (c) Type I and type II keratins complement each other to form obligatory heteropolymers (Lee and Baden, 1976; Franke et al., 1983; Steinert et al., 1984; Hatzfeld and Franke, 1985; Eichner et al., 1986). No isolated keratin is able to form filaments on its own. However, all combinations of equimolar amounts of type I and type II keratin that have been tested assemble into filaments (Hatzfeld and Franke, 1985). The tetrameric subunit of these heteropolymers contains two protein chains of each keratin type as demonstrated by cross-linking experiments (Quinlan et al., 1984; Ward et al., 1985; Eichner et al., 1986). However, it is still not known whether this complementarity between type I and type II keratins occurs at the level of the dimer or of the tetramer; i.e. it is not clear whether the tetramer consists of two identical heterodimers or of a homodimer of a type I and a homodimer of a type II keratin (for review, see Steinert and Roop, 1988). Although cross-linking studies of tetramers have been interpreted to suggest the presence of heterodimers (Quinlan et al., 1984; Ward et al., 1985), these experiments could not discriminate between cross-links occurring within a coiled coil or between neighboring coiled coils of the tetramer (Eichner et al., 1986). Gel filtration of proteolytic products of epidermal keratins also could not distinguish between heterodimers and a mixture of homodimers eluting as a single peak (Parry et al., 1985). On the other hand, individual keratins, as well as their proteolytic fragments, can form homodimers in vitro (Gruen and Woods, 1983; Hatzfeld and Franke, 1985; Quinlan et al., 1986). Moreover, combinations of type I and type II homodimers seem to assemble into heterotetrameric subunits (Hatzfeld et al., 1987; Quinlan et al., 1986) and filaments under special conditions. Assuming that dissociation of homodimeric coiled coils did not take place, the latter experiments were interpreted as suggesting that the tetramer consists of a type I and a type II homodimer. However, since data concerning the stability of coiled coil dimers, their rate of dissociation and exchange of polypeptide chains are not available, the possibility that the tetramer is built according to a heterodimer principle cannot be excluded by these experiments (Hatzfeld et al., 1987).

To analyze the composition of in vitro reconstituted filaments, we have used site specific in vitro mutagenesis with subsequent expression of the mutant proteins in *E. coli* to produce the authentic keratin proteins as well as modified proteins, which allow the introduction of a zero-length cross-link. As a keratin pair we used keratins 18 and 8 since they both lack cysteines (Franz and Franke, 1986; Magin et al., 1986; Singer et al., 1986; Alonso et al., 1987). We have introduced a single cysteine into both keratins in the same position of the coiled coil, since past experience with the tropomyosin coiled coil has shown that a cysteine in such an internal position can readily be oxidized within the coiled coil to form a cystine cross-link (Stewart, 1975; Lehrer, 1975). Using the mutant proteins, we have prepared homo- and heterodimers, containing the cystine cross-link, and analyzed their ability to form IF in vitro. Our results show that heterodimers containing the cross-link can readily assemble into IF that are indistinguishable from nonmutant IF. Mixtures of homodimers stabilized by cystine however do not form normal IF using standard reconstitution conditions.

Materials and Methods

Expression of *Xenopus laevis* Keratin 8 and Mouse Keratin 18 in *E. coli*

Cloning of the cDNA pK XL 1/8 (Franz and Franke, 1986) into the bacterial expression plasmid pINDU (Bujard et al., 1987) has been described (Magin et al., 1987). The murine keratin 18 cDNA clone (Alonso et al., 1987) was kindly provided by Dr. Alonso and was cloned into the Sac I restriction site of M13mpl8. A unique Bam HI site was created at the 5' end of the coding sequence by site specific mutagenesis using the method of Eckstein and co-workers (Nakamaye and Eckstein, 1986; Sayers et al., 1988). The protocol of Amersham Buchler GmbH (Braunschweig, FRG) was followed except that *E. coli* strain JM 101 was used in the transformation reaction. Plaques were screened for mutants by restriction mapping with Bam HI using a standard plasmid miniprep method to prepare rf-DNA from M13mpl8 (Holmes and Quigley, 1981). The mutation introduced was verified by sequence analysis with T7-polymerase (Pharmacia Fine Chemicals, Uppsala, Sweden) using a synthetic oligonucleotide as primer. Rf-DNA prepared from a positive clone using Qiagen pack 100 (Qiagen Inc., Studio City, CA) was cleaved with Sac I and Bam HI and the DNA insert recovered from an agarose gel using gene clean (BIO 101 Inc., La Jolla, CA). DNA of the plasmid pINDU was cleaved with Hind III and Bam HI, and purified by agarose gel electrophoresis. A Hind III-Sac I Adaptor (New England Biolabs, Beverly, MA) was ligated to the Hind III site to create an additional Sac I restriction site. The mouse keratin 18 cDNA was then ligated into pINDU in the correct orientation and reading frame with respect to the AUG start codon of the plasmid. The plasmid construct was then transformed into *E. coli* strain JM 101.

Site-specific Mutagenesis Reactions to Introduce a Cysteine at Position -36 of the Rod

The cDNA of pK XL 1/8 was cloned into M13mpl8 using the unique Bam HI/Hind III sites. 21-mer oligonucleotides with the three base pair mismatch in the middle were used for the mutagenesis reaction. Plaques were screened for mutants by sequence analysis with synthetic oligonucleotides as primers. Rf-DNA was prepared from positive clones, cleaved with Bam HI and Hind III and the insert DNA was recloned into the expression plasmid pINDU. The plasmid was transformed in *E. coli* CMK 603.

M13mpl8, containing the mouse keratin 18 cDNA with the Bam HI restriction site, was used for the mutagenesis reaction to introduce the cysteine. Positive clones were selected by sequence analysis. Cloning of the mutant DNA into the expression plasmid pINDU and expression in *E. coli* JM 101 was done as described above for the nonmutant keratin.

Screening for Keratin Expression

Bacteria were grown overnight in 1-ml minicultures. After centrifugation, bacterial pellets were dissolved in sample buffer (Laemmli, 1970), and proteins analyzed by SDS-PAGE. Polypeptides were transferred to nitrocellulose according to the procedure of Kyhse-Andersen (1984). Keratins were detected in immunoblots (Achtstätter et al., 1986) using the broad specificity IFA-monoclonal antibody (Pruss et al., 1981) and an alkaline phosphatase coupled anti-mouse Ig as second antibody.

Purification of Keratins from Bacteria

Cultures were grown overnight in LB-medium (Maniatis et al., 1982) containing 200 µg/ml ampicillin. Inclusion bodies were purified according to Nagai and Thøgersen (1987) except that high molecular weight DNA was destroyed by shearing in a Dounce homogenizer. Purified inclusion bodies were dissolved in 9.5 M urea, 5 mM EDTA, 1% 2-mercaptoethanol, 10 mM Tris-HCl, pH 8.6 containing the following protease inhibitors: 0.5 µM E64 ([L-3-trans-carboxyoxiran-2-carbonyl]-L-leu-agnatin; Peptide Institute, Osaka, Japan), 100 µg/ml ovomucoid (Sigma Chemical GmbH Munich, FRG) and 2 mM PMSF.

Keratins were purified by ion exchange chromatography on Mono Q (Pharmacia Fine Chemicals) in 8.5 M urea, 5 mM EDTA, 2.5 mM dithioerythritol (DTE), 10 mM Tris-HCl pH 8.6 using a linear gradient from 0 to 200 mM guanidine-HCl. Peak fractions were pooled. After dialysis against 8.5 M urea, 2.5 mM DTE, 10 mM Tris-HCl pH 7.5 samples were applied to an ss-DNA-agarose column (Gibco/BRL Life Technologies Inc.,

Eggenstein, FRG) in the same solvent to bind the keratin (Nelson et al., 1982). Bound keratin was eluted with 200 mM guanidine-HCl.

Preparation of Keratin Homodimers

Keratins purified by ss-DNA-affinity chromatography were dialyzed overnight against 2 M guanidine-HCl, 10 mM Tris-HCl pH 7.5 without any reducing agent to induce dimer formation and air oxidation of the homodimers. To monitor the time course of disulfide formation aliquots were taken, the protein precipitated (Wessel and Flügge, 1984), and analyzed by SDS-PAGE without any reducing agent. Homodimers were separated from residual monomers by gel filtration on Superose 12 (Pharmacia Fine Chemicals) using 2 M guanidine-HCl as solvent.

Preparation of Keratin Heterodimers

Equimolar amounts of keratins 8 (ss-DNA-preparation) and 18 (inclusion body preparation) were combined in 8.5 M urea at a protein concentration of 0.5–1 mg/ml, as measured by the method of Bradford (1976) and dialyzed stepwise for 2–3 h against 5 mM EDTA, 5 mM DTE, 10 mM Tris-HCl pH 8.0, and then against 5 mM EDTA, 5 mM DTE, 50 mM Tris-HCl pH 7.5. Filaments were harvested by centrifugation at 10,000 rpm for 30 min. The pellet was washed twice with 5 mM EDTA, 50 mM Tris-HCl pH 7.5 to remove excess reducing agent and then suspended in 5 mM EDTA, 10 mM Tris-HCl pH 8.0 and incubated for ~3 h at room temperature to allow air oxidation. 10 M urea, 5 mM EDTA, 10 mM Tris-HCl pH 8.5 was added to reach a final urea concentration of 8.5 M. An aliquot was taken to monitor the degree of dimer formation by SDS-PAGE without reducing agent. The bulk of the sample was applied to a Mono Q ion exchange column and eluted with a gradient of 0–200 mM guanidine-HCl.

Reverse Phase HPLC

Keratin dimers were analyzed by reverse phase chromatography using a C4 column (RP-304; Bio-Rad Laboratories, Richmond, CA) and the trifluoroacetic acid/acetonitrile solvent system as described (Quinlan et al., 1984; Hatzfeld et al., 1987). Samples were applied in buffers containing 8.5 M urea. Cyanogen bromide fragments of sperm whale myoglobin (Sigma Chemical Co.) were prepared and used as internal standards to compare the reverse phase chromatograms.

Gel Electrophoresis

One-dimensional SDS-PAGE was performed according to Laemmli (1970). Diagonal electrophoresis was used to characterize the composition of the cross-linked dimers (Quinlan et al., 1986). Samples were dissolved in sample buffer without reducing agent, and separated on 8% polyacrylamide gels (Laemmli, 1970). Relevant gel tracks were excised and incubated in sample buffer containing 1% 2-mercaptoethanol for 30 min at room temperature. Then each gel track was loaded onto a second 8% polyacrylamide gel.

Filament Formation and Electron Microscopy

For reassembly of the nonmutant keratins, equimolar amounts of one keratin purified from *E. coli* and the complementary keratin isolated from rat liver (Achtstätter et al., 1986) were combined in 2 M guanidine-HCl buffer at a protein concentration of 0.5–1 mg/ml and dialyzed against standard filament buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5). The mixture of the nonmutant keratins 8 and 18, both isolated from *E. coli*, was used in the same way.

Cysteine-containing homodimers of keratins 8 and 18 were mixed in equimolar amounts in 2 M guanidine-HCl, the buffer used in purification on Superose S12. The final protein concentration was ~0.5 mg/ml and dialysis was against standard filament buffer without reducing agent. In another experiment homodimers were reduced before reconstitution by addition of 5% 2-mercaptoethanol. The sample was incubated for 30 min at room temperature, and then dialyzed either directly to filament buffer with or without reducing agents, or first to 8.5 M urea buffer followed by filament buffer.

Heterodimers containing the cystine-bridge were dialyzed from 8.5 M urea either directly into standard filament buffer, or first to 2 M guanidine-HCl buffer and then into filament buffer. All solvents were free of reducing agent. Filament formation was monitored by negative staining with 2% uranylacetate.

Results

Expression of Keratins 8 and 18 in *E. coli*

The expression of *Xenopus laevis* keratin 8, a type II keratin, in *E. coli* has been described. Because of the Bam HI restriction site that follows the AUG start codon of the expression plasmid pINDU, the first five amino acid residues of the non- α -helical head domain differ from those in the normal keratin (Magin et al., 1987). However, in contrast to the previously reported sequence the second amino acid is an arginine rather than a glutamic acid. The nucleotide sequence was determined by sequencing the double-stranded plasmid DNA and was ATG AGA and not ATG GAA as reported by Bujard et al. (1987).

To analyze the filament forming ability of keratin homodimers and heterodimers, a type I and a type II keratin were used. As keratins from different species accept each other as promiscuous partners (Hatzfeld and Franke, 1985), we used the cDNA clone of mouse keratin 18 (Alonso et al., 1987) to express a type I keratin. To clone this cDNA into the expression plasmid pINDU, we created a unique Bam HI restriction site in the head domain by site directed in vitro mutagenesis. This mutation allowed the keratin to be cloned into the expression plasmid in the right orientation and reading frame. As shown in Fig. 1 a, the altered nucleotide sequence allows cleavage with Bam HI immediately following the AUG start codon of the authentic keratin 18. The first four amino acids of keratin 18 expressed in *E. coli* differ from the sequence of the normal keratin because of the sequence of the Bam HI restriction site. This change was confirmed by amino acid sequencing of the intact purified keratin.

To screen bacterial cultures for protein expression, total cell extracts separated by SDS-PAGE were subjected to immunoblot analysis. Fig. 2, lane 2, shows that keratin 18 is readily detectable on SDS-gels and reacts with the monoclonal antibody IFA (Fig. 2, lane 2'), which recognizes most IF proteins (Pruss et al., 1981).

Production and Expression of Mutant Keratins 8 and 18

The codon UGU for cysteine was introduced into each of the two cDNA clones via oligonucleotide directed in vitro mutagenesis in M13. In the keratin 8 sequence the alanine of position -36 of the rod domain was substituted by cysteine (Fig. 1 b). In the same relative position of keratin 18 the threonine was replaced (Fig. 1 b). The mutant keratins are called XK8/cys (*Xenopus* keratin 8/cysteine-containing mutant) and MK18/cys (mouse keratin 18/cysteine-containing mutant). Both mutant cDNAs were cloned into the expression plasmid using the Bam HI restriction site. Both cysteine mutants were detected by the IFA-monoclonal antibody (Fig. 2, lanes 3' and 5'). For unknown reasons protein expression in *E. coli* was higher for both mutant keratins than for their unmutated counterparts (Fig. 2, lanes 2, 3, and 5). When samples prepared without 2-mercaptoethanol were separated on gels, some homodimers were detected in the immunoblots (Fig. 2, lane 3').



Figure 1. (a) Comparison of the nucleotide and amino acid sequences of the 5'-coding region of the authentic mouse keratin 18 and mouse keratin 18 synthesized in *E. coli*. The N-terminal amino acid sequence M-R-G-S is determined by the AUG start codon of the expression plasmid pINDU that is followed by a Bam HI restriction site as indicated. The sequence AGCTTC coding for the amino acids S-F in the authentic mouse keratin 18 was changed to the Bam HI restriction site GGATCC by site directed in vitro mutagenesis. T-T-R is then the start of the amino acid sequence common to both proteins. For corresponding documentation of the recombinant *Xenopus* keratin 8, see Magin et al., 1987. (b) Amino acid sequences of the mutant cyto keratins XK8/cys and MK18/cys. Coils 1A, 1B and 2 indicate the segments of the α -helical rod domains. The dots mark the positions of hydrophobic amino acids in positions *a* and *d* of the coiled coil heptad repeat pattern. The arrows indicate the positions of the cysteines that were introduced by site directed in vitro mutagenesis. The reference point of alignment is the consensus sequence at the end of the rod domain. The arrowhead indicates reference position 0. Both mutant proteins carry the cysteine in position -36, which is a *d* position. (For original sequences, see Franz and Franke, 1986; Alonso et al., 1987.) These sequence data are available from EMBL/GenBank/DBJ under accession number A02953 and M11686.

Purification of Keratins from *E. coli* and Homodimer Formation by the Mutant Proteins

The nonmutated keratins, as well as the cysteine containing mutant proteins, were isolated as inclusion bodies according to the method of Nagai and Thogersen (1987). As judged by SDS-PAGE (Fig. 3 *a*, lane 4; Fig. 3 *b*, lane 2), such preparations were highly enriched in keratins (~80–90% of the total protein). Mono Q anion exchange chromatography and ss-DNA affinity chromatography both in 8.5 M urea were used to further purify the nonmutant keratins 8 and 18.

Inclusion body preparations of the mutant keratins were directly subjected to ss-DNA chromatography in 8.5 M urea (Fig. 3 *a*, lane 5, and Fig. 3 *b*, lane 3). Samples were subsequently dialyzed into 2 M guanidine-HCl buffer in the absence of reducing agents to allow air oxidation of the cysteines, and to induce homodimer formation (Quinlan et al., 1986). The yield of cross-linked dimers was consistently >50% and usually approached ~75% (Fig. 3 *a*, lane 6). Gel filtration in 2 M guanidine-HCl on Superose 12 separated the cross-linked dimer from residual monomer. Thus, a pure

homodimer fraction was obtained for both mutant proteins (Fig. 3 *a*, lane 7, and Fig. 3 *b*, lane 4).

Heterodimer Formation in the Intact Filaments; Purification

MK18/cys, from inclusion body preparations (Fig. 4 *b*, lane 1), and XK8/cys, from ss-DNA agarose (Fig. 4 *b*, lane 2), were mixed in 9.5 M urea containing 1% 2-mercaptoethanol (Fig. 4 *b*, lane 3). This mixture of reduced and denatured monomers was allowed to renature by stepwise dialysis, first into 10 mM Tris-HCl (pH 8), and then into 50 mM Tris-HCl buffer pH 7.5 (see Materials and Methods). Filaments formed in the presence of reducing agent were harvested by centrifugation and resuspended in 50 mM Tris-HCl pH 7.5 buffer lacking reducing agent to allow air oxidation. Within 3 h, at least 50% of the keratins were cross-linked (Fig. 4 *b*, lane 4). Although the yield of cystine containing heterodimer (see below) increased with longer incubation times to at least 90%, a 3-h incubation time was routinely used to minimize proteolytic degradation, which occurred only in the absence

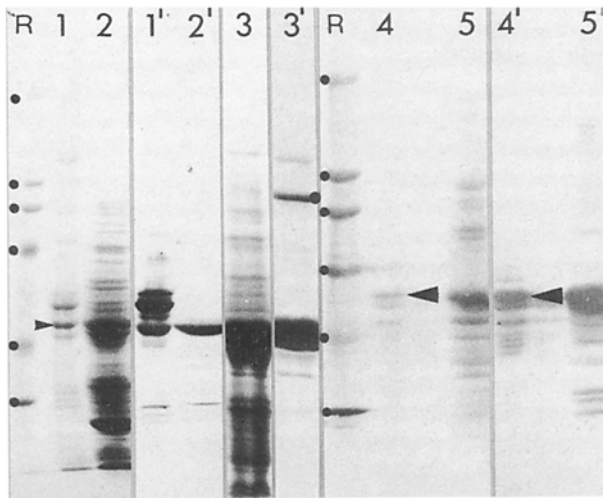


Figure 2. SDS-PAGE and immunoblot analysis of mouse keratin 18, mouse keratin 18/cys and *Xenopus laevis* keratin 8/cys expressed in bacteria. Lanes 1 and 1', cytoskeletal fraction derived from mouse liver. The arrowhead denotes the position of authentic mouse keratin 18. Lanes 2 and 2', total extract from bacteria expressing mouse keratin 18. Lanes 3 and 3', total extract from bacteria expressing MK18/cys. The dot in lane 3' denotes cross-linked MK18/cys dimer. Lanes 4 and 4', cytoskeletal proteins from *Xenopus laevis* A6 cells. The arrowhead denotes the position of the authentic *Xenopus laevis* keratin 8. Lanes 5 and 5', total extract from bacteria expressing XK8/cys. Lane R, reference proteins with molecular weights of 205,000 (myosin), 116,000 (β -galactosidase), 97,400 (phosphorylase b), 66,000 (BSA), 45,000 (ovalbumin), and 29,000 (carbonic anhydrase). Lanes 1–5 and R are stained with Ponceau S after separation of polypeptides by SDS-PAGE and transfer to nitrocellulose. Lanes 1'–5' show the corresponding immunoblot reaction after incubation with monoclonal antibody IFA (Pruss et al., 1981) and an alkaline phosphatase-coupled second antibody.

of urea (Fig. 4 b, lane 4). Oxidative cross-linking was stopped by denaturation in 8.5 M urea. Dimers containing the cystine cross-link were separated from XK8/cys and MK18/cys monomers and from degradation products by Mono Q anion exchange chromatography in 8.5 M urea (Fig. 4 a). Fractions were analyzed by SDS-PAGE under nonreducing (Fig. 4 b, lanes 5–16), and reducing conditions (Fig. 4 b, lanes 5'–16'). Electrophoresis under nonreducing conditions showed that monomeric XK8/cys eluted first from the column (Fig. 4 b, lanes 5–8). It was followed by monomeric MK18/cys (Fig. 4 b, lanes 9–11). The major peak fraction contained nearly exclusively dimer (Fig. 4 b, lanes 12 and 13), while the subsequent shoulder (fractions 14–16 in Fig. 4 a) revealed dimer and some degradation products (Fig. 4 b, lanes 14–16). In SDS-PAGE, under reducing conditions, the dimer band separated into XK8/cys and MK18/cys monomer (Fig. 4 b, lanes 12' and 13'). The very clear separation of the two distinct monomers on the Mono Q column suggested that the following sharp dimer peak should be a heterodimer and not a mixture of two homodimers (for direct proof see below). Fractions containing degradation-free dimer and very little MK18/cys monomer were used for the further characterization (Fig. 4 b, lanes 12 and 13).

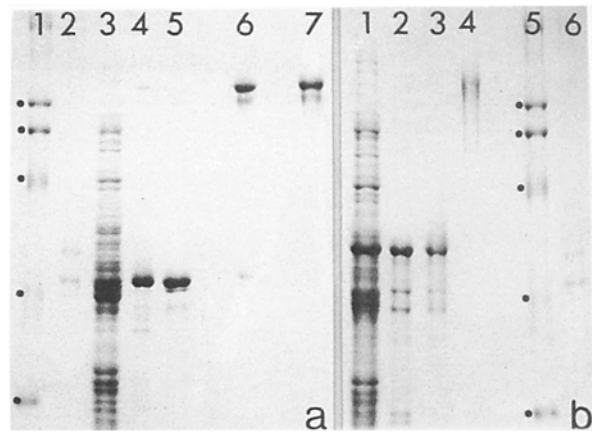


Figure 3. (a) SDS-PAGE demonstrating the purification of MK18/cys. Lane 1, reference proteins β -galactosidase (116,000), phosphorylase b (97,400), BSA (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000). Lane 2, keratins 8 and 18 extracted from mouse liver. Lane 3, total extract from bacteria expressing MK18/cys. Lane 4, preparation of inclusion bodies from the same bacterial culture. Lane 5, MK18/cys after purification by ss-DNA affinity chromatography. Lane 6, same sample as in lane 5 after dialysis to 2 M guanidine-HCl buffer without reducing agent. Note the prominent dimer band. Lane 7, purified MK18/cys dimer after Superose 12 gel filtration. Lanes 6 and 7 were analyzed without reducing agent in the sample buffer. (b) SDS-PAGE showing the purification of XK8/cys. Lane 1, total extract of bacteria expressing XK8/cys. Lane 2, inclusion bodies prepared from the same bacterial culture. Lane 3, sample of XK8/cys after ss-DNA affinity chromatography. Lane 4, cystine-dimer of XK8/cys after purification by Superose 12 gel chromatography. Lane 5, reference proteins as in a. Lane 6, authentic keratins 8 and 18 from mouse liver. The sample in lane 4 was analyzed in the absence of a reducing agent.

Characterization of the Cystine-containing Heterodimer

The presumptive heterodimer fraction from the Mono Q chromatography (e.g., fractions 12 and 13 in Fig. 4) was further analyzed by diagonal electrophoresis. Fig. 5 gives the summary of several experiments. Lane 2 of Fig. 5 a shows a mixture of XK8/cys homodimer and MK18/cys homodimer, which separate into two bands in one-dimensional SDS-PAGE under nonreducing conditions. The presumptive heterodimer isolated by Mono Q chromatography, however, migrates predominantly as a single band (Fig. 5 a, lane 3). Reduction of the cystines with 2-mercaptoethanol and diagonal electrophoresis of the cleaved products in the second dimension shows that the dimers from Mono Q contain equal amounts of XK8/cys and MK18/cys (Fig. 5 c). The migration of the two proteins on the same vertical line indicates that they formed one cross-linked product before reduction. The reduction of homodimer bands, however, indicates that the upper band represents the XK8/cys homodimer, whereas the lower band contains MK18/cys homodimers (Fig. 5 b).

Filament Formation by the Cystine Heterodimer

We first explored whether keratins with a slightly altered N-terminal sequence that was generated by the cloning protocol can still assemble into normal IF. We reconstituted each of

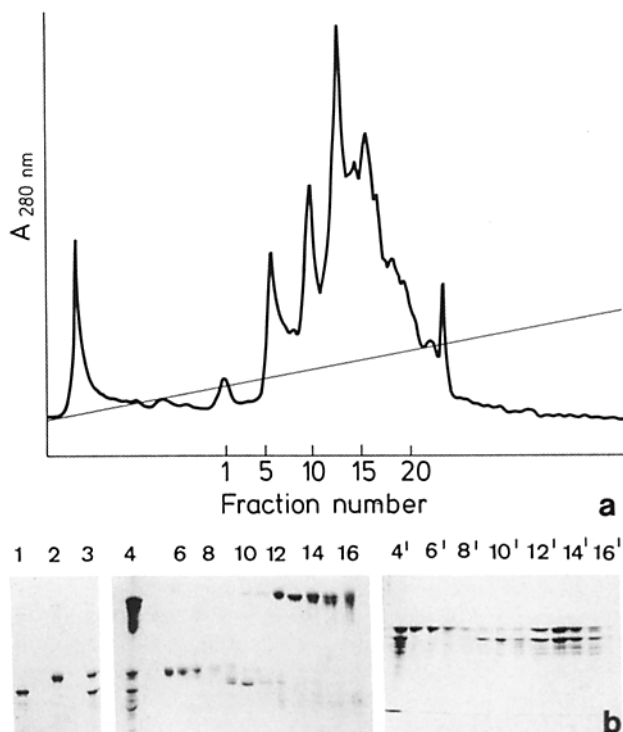


Figure 4. Purification of XK8/cys and MK18/cys heterodimer containing the cystine bond. Filaments formed by the reduced monomers were harvested and allowed to air oxidize. Filaments were dissolved in 8.5 M urea (see Materials and Methods) and the protein species analyzed. (a) Elution profile of Mono Q anion exchange chromatography in 8.5 M urea, 5 mM EDTA, 10 mM Tris-HCl (pH 8.5) with a gradient from 0 to 80 mM guanidine-HCl. Absorption was monitored at 280 nm. Major peak fractions were collected as indicated. (b) Analyses of fractions from Mono Q chromatography by SDS-PAGE under nonreducing (lanes 1-16) and reducing (lanes 4'-16') conditions. Lane 1, MK18/cys inclusion bodies. Lane 2, XK8/cys from ss-DNA agarose. Lane 3, equimolar mixture of XK8/cys and MK18/cys in 8.5 M urea buffer used as starting material to induce heterodimer formation. Lanes 4 and 4', aliquots of the same sample as in lane 3 after filament formation and air oxidation separated under nonreducing (lane 4) and reducing (lane 4') conditions. This sample was subjected to Mono Q chromatography. Lanes 5-16, fractions from Mono Q chromatography as indicated in a with XK8/cys monomers present in fractions 5-8. MK18/cys monomers are found in fractions 9-12 while XK8/cys MK18/cys dimers elute in fractions 12-16. Fractions 12 and 13 contain a single dimer band whereas fractions 14-16 contain also a series of proteolytic degradation products of the dimer. Lanes 5'-16', corresponding samples analyzed in the presence of 2-mercaptoethanol.

the nonmutant keratins with an authentic keratin of the complementary subfamily isolated from rat liver (Achtstätter et al., 1986). Fig. 6 a shows negatively stained IF using recombinant *Xenopus laevis* keratin 8 and rat liver keratin 18, while Fig. 6 b shows reconstituted IF containing recombinant murine keratin 18 and rat liver keratin 8. Both samples reveal typical IF. The combination of the two recombinant keratins also assembled readily into IF, morphologically indistinguishable from liver IF (Fig. 6 c). Therefore, we next studied the reconstitution of the cystine heterodimer between the two mutant keratins and the mixture of the two cystine containing homodimers using dialysis into standard filament buffer

without reducing agent. Fig. 7 shows the reconstitution of IF from heterodimer obtained by Mono Q chromatography. The cystine heterodimer readily assembles into typical IF. No morphological difference can be detected between filaments formed in the absence (Fig. 7), or in the presence, of reducing agents (not shown). In contrast, the combination of the two homodimers, each stabilized by a cystine bond, was unable to form IF using standard reconstitution conditions. Instead very large aggregates are formed (Fig. 8 a), which sometimes seem connected via fibrillar material (Fig. 8 a, arrows). Interestingly, however, when 2-mercaptoethanol was added the homodimer mixture readily formed filaments (Fig. 8 b). These experiments suggest that homodimers with an internal cystine cross-link cannot form IF unless the cross-link is opened to allow dissociation and subsequent heterodimer formation (see also below).

Biochemical Characterization of IF Reassembled from the Mutant Keratins

To rule out the possibility that a very small subpopulation, comprising non-cross-linked keratins in the heterodimer sample, could be responsible for IF formation documented by electron microscopy, an analysis similar to that presented in Fig. 5, a-c was also applied to the reconstituted filaments. Aliquots of samples used for electron microscopic analysis were centrifuged and the pellet and supernatant were analyzed by one-dimensional SDS-PAGE (Fig. 5 d, lanes 2 and 3). The supernatant fraction of filaments reconstituted from cross-linked heterodimers did not reveal protein in detectable amounts (Fig. 5 d, lane 2), indicating that filaments were obtained in very good yield. The corresponding pellet fraction showed one major band (Fig. 5 d, lane 3). This was again subjected to diagonal electrophoresis to identify the constituents of the cross-linked products. Since after reduction XK8/cys and MK18/cys were identified on the same vertical line, the filaments did contain heterodimers (Fig. 5 f).

The large aggregates that were visible electron microscopically (Fig. 8 b) and resulted from a mixture of XK8/cys homodimers and MK18/cys homodimers were harvested by centrifugation, and also analyzed by diagonal electrophoresis. In the first dimension, two bands representing XK8/cys homodimers and MK18/cys homodimers were separated (Fig. 5 d, lane 1). Diagonal electrophoresis revealed that the upper and lower bands contain XK8/cys and MK18/cys, respectively (Fig. 5 e).

Cross-linked hetero- and homodimers were further characterized by analytical reverse phase HPLC. When filaments originating from cystine containing heterodimers (Fig. 7, a and b) were dissolved in 8.5 M urea, lacking reducing agent, and applied to the reverse phase column, a single peak was obtained (Fig. 9 a). It contained only one dimer band in SDS-PAGE without reducing agent (Fig. 9 d, lanes 1-3). However, when analyzed on gels with 2-mercaptoethanol (Fig. 9 d, lanes 1'-3'), both XK8/cys and MK18/cys were documented. In contrast, the reconstituted mixture of XK8/cys and MK18/cys homodimers (see Fig. 8 b) was separated by HPLC into two peaks (Fig. 9 b), which represent cross-linked homodimers of MK18/cys (Fig. 9 d, lanes 4 and 4') and XK8/cys (Fig. 9 d, lanes 6 and 6'), respectively.

Filaments obtained from reduced XK8/cys and MK18/cys homodimers (see Fig. 8 a) were oxidized by dialysis into filament buffer without reducing agent, harvested by centrifuga-

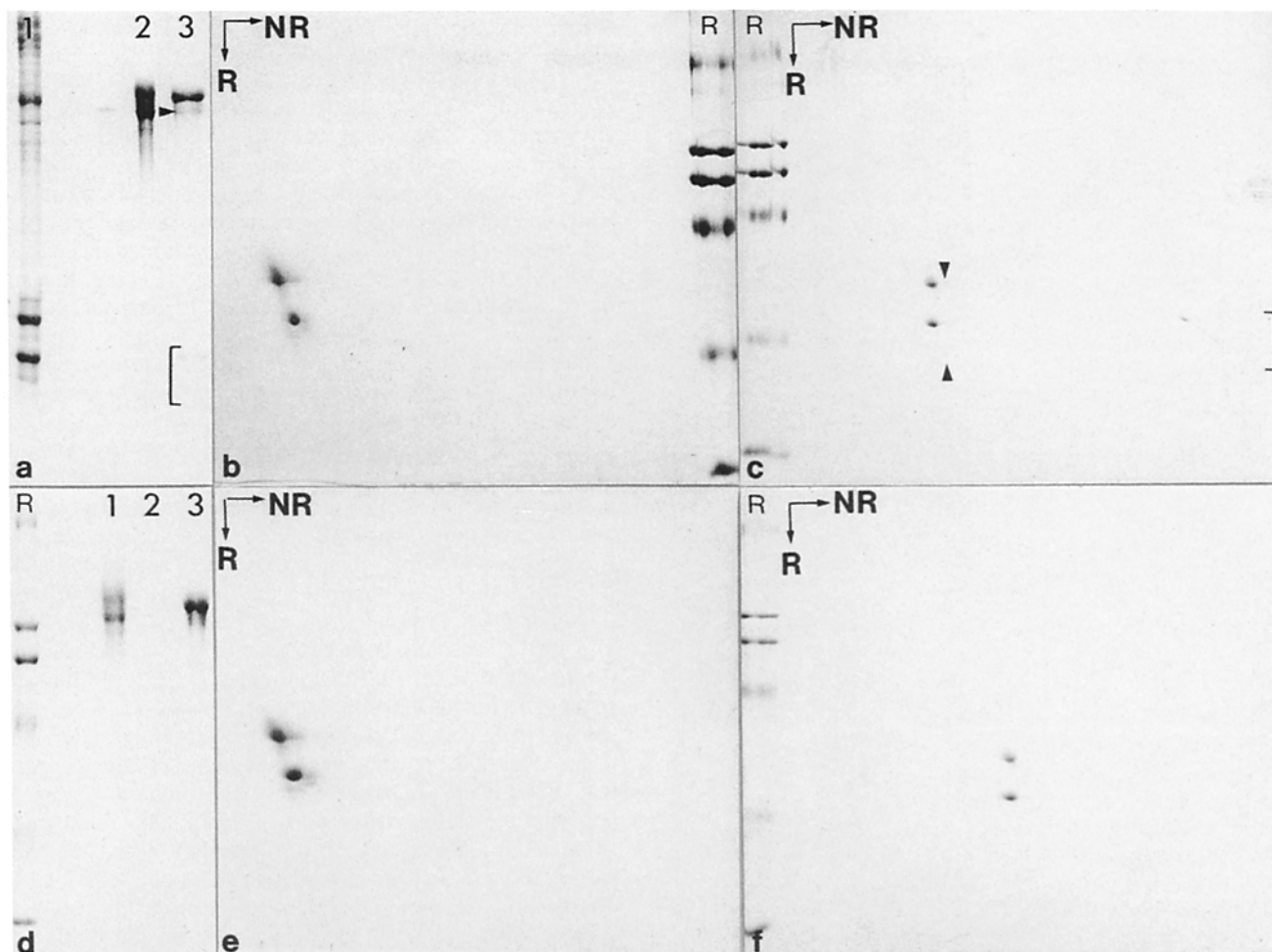


Figure 5. Electrophoretic characterization of cross-linked heterodimer between XK8/cys and MK18/cys. (a) One-dimensional SDS-PAGE (8% gels) showing a mixture of XK8/cys and MK18/cys homodimers from gel filtration chromatography (lane 2) and the heterodimer of XK8/cys and MK18/cys isolated by Mono Q chromatography (lane 3). The arrowhead denotes a degradation product of heterodimer; the bracket indicates some residual non-cross-linked MK18/cys (see also Fig. 4 b, lanes 12 and 13). Lane 1, keratins 8 and 18 from mouse liver. The gel was run under nonreducing conditions. (b) Diagonal gel electrophoresis of the sample shown in a, lane 2 (mixture of homodimers). Cross-links were cleaved by incubation with 2-mercaptoethanol. NR, direction of first dimension (electrophoresis under nonreducing conditions). R, direction of second dimension (electrophoresis under reducing conditions). Reference proteins are as in Fig. 2. Monomeric XK8/cys was released from the upper dimer band and monomeric MK18/cys from the lower dimer band. (c) Diagonal electrophoresis of the heterodimer shown in a, lane 3. Monomeric XK8/cys and MK18/cys migrate on the same vertical indicating that they have been included in one cross-link product in the first electrophoresis, which was done under nonreducing conditions. The arrowheads denote the product of the heterodimeric degradation band containing XK8/cys and the rod fragment of MK18/cys. The bracket denotes monomeric MK18/cys and some degradation products that have not been cross-linked in the first dimension electrophoresis and therefore migrate on a diagonal. (d) One-dimensional SDS-PAGE of reassembled homodimers and heterodimer. Lane 1, XK8/cys and MK18/cys homodimers dialyzed against standard filament buffer (see Materials and Methods). The aberrant aggregates formed in this buffer (see Results) were pelleted by centrifugation, and dissolved in sample buffer without reducing agent. Lanes 2 and 3, IF reassembled from cross-linked heterodimer were harvested by centrifugation, and dissolved in sample buffer under nonreducing conditions (lane 3). Proteins from the supernatant were precipitated and also analyzed under nonreducing conditions (lane 2). Note the virtual absence of protein in the supernatant. (e) Diagonal electrophoresis of the sample shown in d, lane 1 (aberrant aggregates formed by cross-linked homodimers). XK8/cys monomers are released from the upper dimer band, MK18/cys monomers from the lower dimer band. Thus, the large aggregates are made of the two cystine stabilized homodimers. (f) Diagonal electrophoresis of the IF pellet shown in d lane 3 (IF from heterodimer). XK8/cys and MK18/cys monomers migrate at the same vertical after reduction in the second dimension electrophoresis.

tion and dissolved in 8.5 M urea without reducing agent. The elution profile from the HPLC column identified the major peak (Figs. 9, c and d, lanes 7 and 7') as a heterodimer. The shoulder contained XK8/cys monomer (Fig. 9 d, lanes 8 and 8') and the small subsequent peak was identified as MK18/cys monomer (Fig. 9 d, lanes 9 and 9'). This analysis suggests that homodimers, when not stabilized by a cystine, can

rearrange to heterodimers, which subsequently assemble into filaments.

Discussion

Keratin IF are obligatory heteropolymers, but it is not clear at which level of assembly the obligatory requirement for

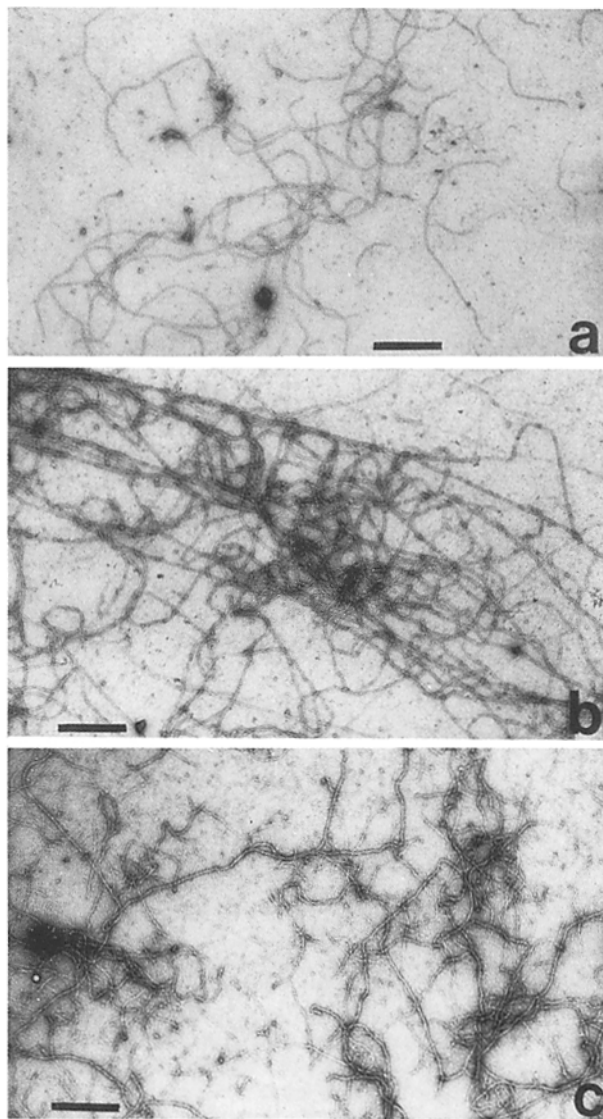


Figure 6. Electron microscopy showing IF assembled from purified keratins expressed in *E. coli*. Purified keratins 8 and 18 were mixed in 8.5 M urea and dialyzed against 50 mM Tris-HCl buffer pH 7.5. Samples were negatively stained with 2% uranylacetate. (a) IF containing *Xenopus laevis* keratin 8 expressed in *E. coli* and keratin 18 from rat liver. (b) IF assembled from mouse keratin 18 expressed in *E. coli* and authentic keratin 8 from rat liver. (c) IF formed by recombinant *Xenopus laevis* keratin 8 and recombinant mouse keratin 18. Bars, 0.5 μm .

two distinct protein chains occurs (for review, see Steinert and Roop, 1988), and whether a polymorphism can occur at the dimer level (Steinert et al., 1989). Studies using keratin fragments obtained by limited proteolysis suggested the existence of hetero coiled coils in two systems (Woods and Inglis, 1984; Parry et al., 1985; Sparrow et al., 1989; Steinert et al., 1989), but the coexistence of hetero- and homodimers has been inferred for a third system (Steinert et al., 1989). Since homodimers can be readily formed in vitro, it has been suggested that they also may directly polymerize into filaments (Quinlan et al., 1986; Hatzfeld et al., 1987), particularly as theoretical calculations based on ion pair formation

did not reveal a difference in the relative stabilities of homo and hetero coiled coils (Steinert et al., 1984). Therefore, several attempts have been made to analyze the keratin complementarity by chemical cross-linking (Quinlan et al., 1984; Eichner et al., 1986; Ward et al., 1986). Since all these studies used reagents acting on ϵ amino groups and did not further chemically characterize the residues involved, an unequivocal conclusion could not be drawn. As ϵ amino groups are common in keratin rod sequences such methods cannot distinguish intra and inter coiled coil cross-linking. In addition, cross-linking outside the α -helical regions was not excluded.

In this study, we describe a novel approach using recombinant DNA technology to analyze the first step in keratin IF assembly; i.e. the formation of the dimeric coiled coil, by chemical cross-linking. However, in contrast to previous studies, we introduced a zero-length cross-link in a defined position within the coiled coil dimer molecule. The cDNA clones for keratins 8 and 18 were chosen for two reasons. First, the two keratins, which are typical of simple epithelia, have been shown to copolymerize into filaments in vitro (Quinlan et al., 1984; Hatzfeld and Franke, 1985; Magin et al., 1987). Second, both proteins lack cysteine residues (Franz and Franke, 1986; Alonso et al., 1987). In the first set of experiments the nonmutant keratins 8 and 18 were expressed in *E. coli* and purified from bacterial lysates. Since the mixture of the purified recombinant proteins formed normal IF, we made use of site directed in vitro mutagenesis to introduce a cysteine in both proteins in the same relative position of the α -helical rod. Thus, keratin homo- and heterodimers could be stabilized by oxidative disulfide formation and dissociation of preformed dimers before or during polymerization was prevented. Our results confirm the finding of Quinlan et al. (1986) that homodimers are formed in 2 M guanidine-HCl. We used this solvent to prepare the two cysteine containing homodimers in good yields. The homodimers were further purified by gel filtration. However, contrary to previous speculations, the mixture of homodimers with their cysteine bond intact was unable to form IF using the standard reconstitution conditions. When the 2 M guanidine-HCl solution was dialyzed against IF assembly buffer lacking a reducing agent, only large aggregates of aberrant morphology were formed. An entirely different situation was observed when the heterodimer containing the cysteine bridge was used. This was prepared by assembling IF from the mixture of reduced monomers and then allowing the filaments to oxidize. Upon subsequent dissociation in 8.5 M urea, only a single type of dimer was observed, which was formed in very high yield. Diagonal electrophoresis and reverse phase HPLC identified this dimer as the cysteine containing heterodimer. When the pure heterodimer was dialyzed from 2 M guanidine-HCl into assembly buffer lacking reducing agent, it again formed morphologically normal IF.

Our results show that the obligatory heteropolymeric assembly of keratin filaments in vitro has a very strong preference for heterodimeric coiled coils rather than the mixture of the two homodimers. In addition, we describe a previously unexpected property of homodimers that has clearly complicated earlier analyses. In 2 M guanidine-HCl, homodimers, which are not stabilized by the cysteine bond, behave as metastable species. In the presence of the complementary homodimer, subunit exchange leads to the heterodimer and/

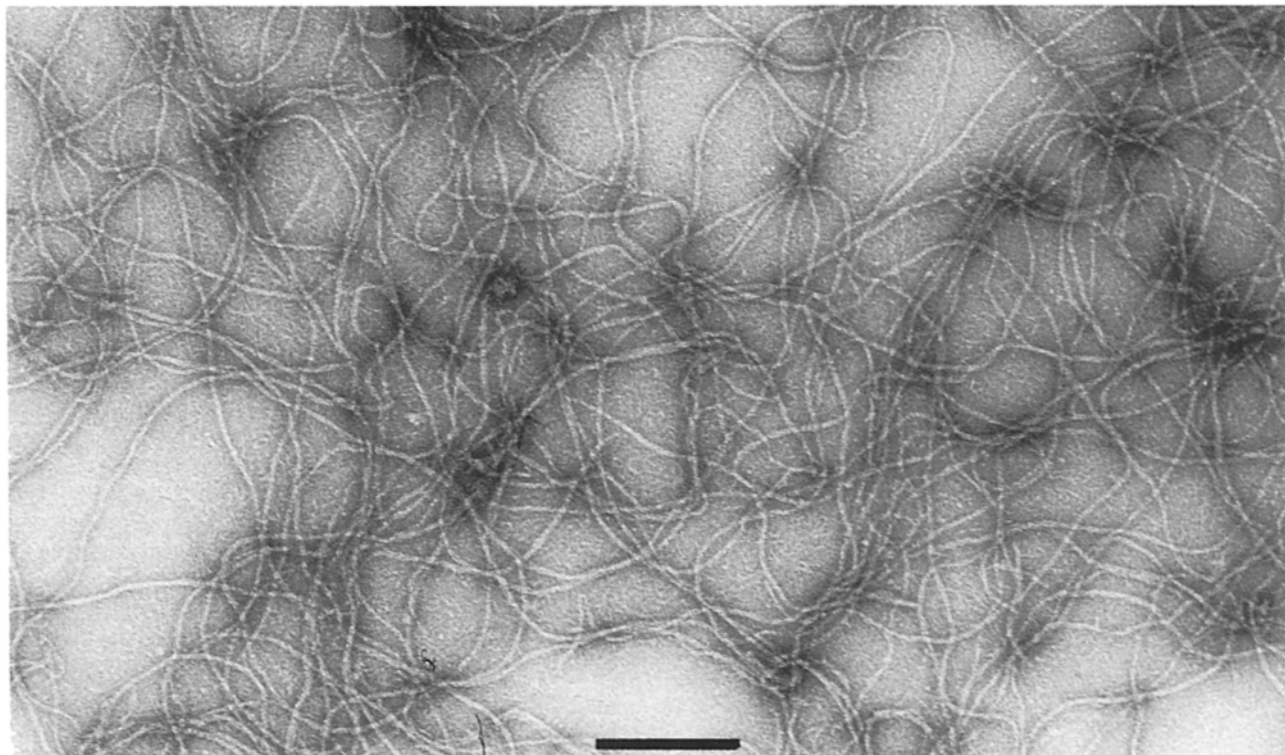
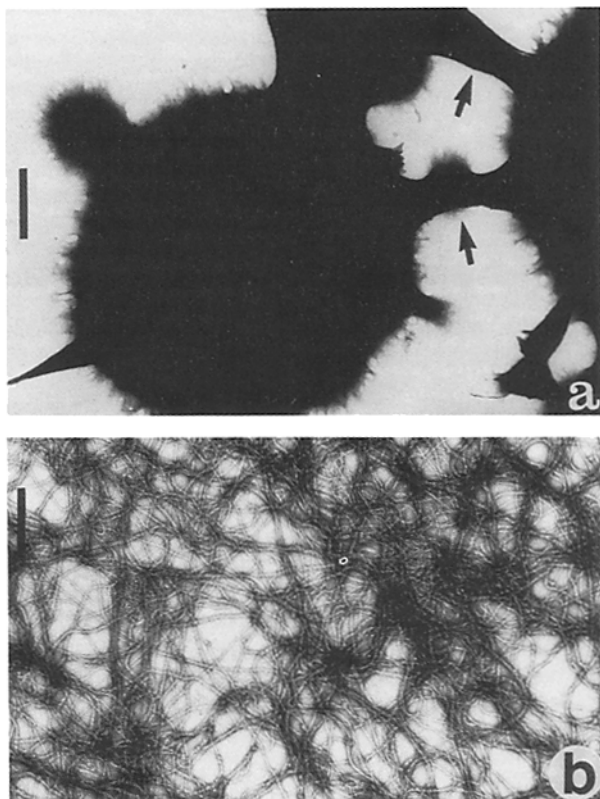


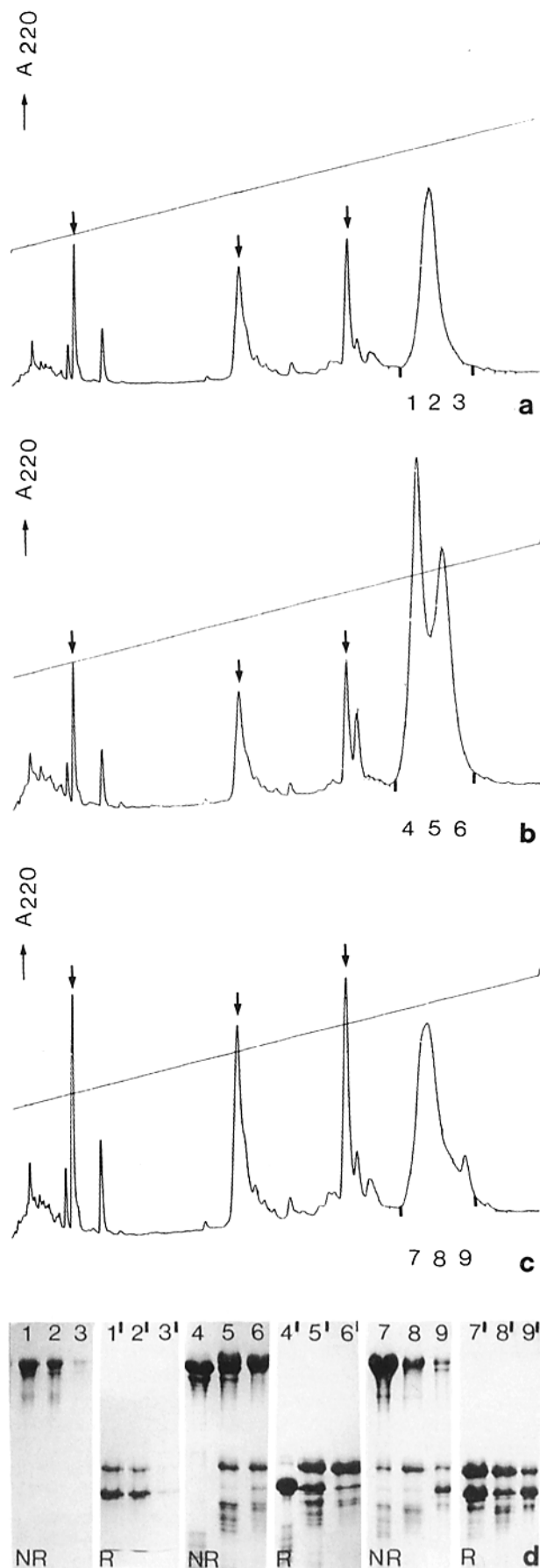
Figure 7. Electron micrograph of IF assembled from the cystine-linked heterodimer between XK8/cys and MK18/cys purified by Mono Q anion exchange chromatography. Fractions containing heterodimers (e.g., Fig. 4 *b*, lanes 12 and 13) were dialyzed to standard filament buffer without reducing agent. Aliquots of the samples were negatively stained. The preparation shows the typical and very long IF. Bar, 0.3 μm .



or heterotetramer, which appear to be the energetically favored forms. The heterodimer then forms filaments in assembly buffer.

Since homodimers exist in 2 M guanidine-HCl, it was earlier thought that IF formation from the two distinct homodimers most likely retains the original state of the molecules (Quinlan et al., 1986; Hatzfeld et al., 1987). Use of the mutant keratins seems to exclude this interpretation. When homodimers are fixed in the cystine form, IF formation does not occur. However, IF formation is possible as soon as the disulfide bond is opened by a reducing agent. The fact that

Figure 8. Electron microscopy of samples containing equimolar mixtures of XK8/cys homodimers and MK18/cys homodimers, each stabilized by a cystine bond. (a) Cross-linked homodimers purified by gel filtration were mixed and directly dialyzed from 2 M guanidine-HCl to filament buffer without addition of reducing agent. The negatively stained preparation shows large aberrant aggregates interconnected by their fibrous structures (arrows) that are clearly different from normal IF. Bar, 0.5 μm . (b) The same homodimer mixture in 2 M guanidine-HCl was treated with 5% 2-mercaptoethanol for 30 min before dialysis against standard filament buffer. The negatively stained sample shows typical long IF. Bar, 0.3 μm . Note that homodimers stabilized by the cystine bond do not form IF (a) unless the bond is opened (b). Analysis of these IF (Fig. 9) shows that they arose by subunit exchange leading to heterodimers.



dissociation of homodimers and subsequent heterodimer formation takes place is clearly shown by the analysis of filaments formed from the two reduced homodimers. Air oxidation after polymerization of IF revealed that again only one cross-link product, the heterodimer, had formed.

As conditions that cause keratin IF to disassemble into stable dimers have not been described so far, we cannot decide whether the heterodimeric coiled coil molecule of a type I and a type II keratin is more stable than the coiled coil of a single keratin, or whether stabilization occurs upon formation of the heterotetramer, which may already take place in the 2 M guanidine-HCl buffer. Theoretical calculations of stabilities of homodimeric versus heterodimeric coiled coils seem to reveal no preference for either of the two forms (Steinert et al., 1984). Circular dichroism spectra in 2 M guanidine-HCl point to a decrease in helix stability for homodimers of keratin 18 in comparison to homodimers of keratin 8 (Quinlan et al., 1986). However, the stability of the heterodimer could not be determined under the same conditions as equimolar combinations of type I and type II polypeptides assemble into tetramers in 2 M guanidine-HCl. This complex formation leads to a strong increase in helix stability (Gruen and Woods, 1983), suggesting that the more important step of stabilization is the heterotetramer formation. Moreover, the predominant subunit structure of most IF proteins except for lamins (Aebi et al., 1986) *in vivo* as well as *in vitro* is thought to be the tetramer rather than the dimer (Geisler and Weber, 1982; Quinlan et al., 1984; Ip et al., 1985; Soellner et al., 1985). Although dimers of des-

Figure 9. Characterization of reconstituted mutant keratins by reverse phase HPLC. Keratin filaments were reconstituted by dialysis into filament buffer. Polymers were harvested by centrifugation, dissolved in 8.5 M urea lacking reducing agent (see text) and applied to a C4 reverse phase column. Proteins were eluted with a linear gradient from 30–55% acetonitrile as indicated. Absorption was monitored at 220 nm. (a) Elution profile of the heterodimer between XK8/cys and MK18/cys containing a cystine bond. The arrows mark the cyanogen bromide fragments of myoglobin that were added to each run as an internal standard. Fractions from the single dimer peak were collected as indicated and analyzed by one-dimensional SDS-PAGE (d) under nonreducing conditions (NR, lanes 1–3) and reducing conditions (R, lanes 1'–3'). (b) Elution profile of the reconstituted mixture of homodimers of XK8/cys and MK18/cys. Each homodimer is stabilized by a cystine bond. Arrows denote the internal standards. The homodimer mixture eluted in two separate peaks; fractions were collected as indicated. (c) SDS-PAGE under nonreducing and reducing conditions shows that the first peak contains the MK18/cys homodimer (lanes 4 and 4') whereas the second peak contains predominantly the XK8/cys homodimer (lanes 6 and 6'). (d) IF formed by a mixture of reduced homodimers were harvested and allowed to oxidize. The elution profile characterizes the dimer type formed after oxidation. Arrows denote the internal standard as in a and b. One major peak fraction was obtained and fractions collected as indicated. In SDS-PAGE (d) the proteins from the major peak fraction migrate as a heterodimer under nonreducing conditions (lane 7). XK8/cys and MK18/cys monomers are detected after reduction (lane 7'). The small shoulder peak (fraction 8) contains the heterodimer and XK8/cys monomer (lanes 8 and 8') whereas the small peak fraction 9 contains predominantly MK18/cys monomer (lanes 9 and 9').

min could be obtained by depolymerization of IF in 3 M guanidine-HCl, circular dichroism studies indicated that the α -helix was already partly destroyed under these conditions (Quinlan et al., 1986). Thus, these isolated dimers do not represent intact α -helical coiled coil molecules.

The molecular nature of the keratin dimer in the filament has also been studied by the physical and chemical properties of domains proteolytically excised from the filaments. When this method was applied to native murine epidermal keratin filaments Steinert et al. (1989) found heterodimers as the sole structural unit. Interestingly, when the same material was polymerized *in vitro* the resulting filaments seemed to contain heterodimers and homodimers in nearly equal amounts. Thus, heterodimers are deduced for *in vivo* formed epidermal keratin filaments by the proteolysis procedure (Steinert et al. 1989) and for *in vitro* assembled keratin filaments using recombinant keratins 8 and 18 (see Results). However, the unexpected permissiveness during *in vitro* filament formation by epidermal keratins (Steinert et al. 1989) is currently not understood, since, in our *in vitro* analysis of recombinant keratins 8 and 18, an equimolar mixture of the two cystine-fixed homodimers provided essentially aberrant aggregates (Fig. 8). Future experiments have to decide whether the reported permissiveness is a peculiarity of epidermal keratins *in vitro*, possibly influenced by details of the assembly process that are not obvious at this stage. While such permissiveness may be related to a polymorphism possibly because of improper alignments of chains (Steinert et al., 1989) the molecular basis for this phenomenon is not yet understood. Finally, we note an observation made with wool α -keratins. Limited chymotryptic digestion of the reduced and carboxymethylated wool microfibrillar complex leads to excision of a tetrameric particle composed of coil 1b segments. A by-product of this reaction arising by transpeptidation strongly suggests that the coiled coil dimer is a heterodimer (for review, see Sparrow et al., 1989).

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