Residues in the 1A Rod Domain Segment and the Linker L2 Are Required for Stabilizing the A11 Molecular Alignment Mode in Keratin Intermediate Filaments*

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Both analyses of x-ray diffraction patterns of well oriented specimens of trichocyte keratin intermediate filaments (IF) and in vitro cross-linking experiments on several types of IF have documented that there are three modes of alignment of pairs of antiparallel molecules in all IF: A11, A22 and A12, based on which parts of the major rod domain segments are overlapped. Here we have examined which residues may be important for stabilizing the A11 mode. Using the K5/K14 system, we have made point mutations of charged residues along the chains and examined the propensities of equimolar mixtures of wild type and mutant chains to reassemble using as criteria: the formation (or not) of IF in vitro or in vivo; and stabilities of one- and two-molecule assemblies. We identified that the conserved residue Arg10 of the 1A rod domain, and the conserved residues Glu4 and Glu8 of the linker L2, were essential for stability. Additionally, conserved residues Lys31 of 1A and Asp1 of 2A and non-conserved residues Asp/Asn9 of 1A, Asp/Asn3 of 2A, and Asp7 of L2 are important for stability. Notably, these groups of residues lie close to each other when two antiparallel molecules are aligned in the A11 mode, and are located toward the ends of the overlap region. Although other sets of residues might theoretically also contribute, we conclude that these residues in particular engage in favorable intermolecular ionic and/or H-bonding interactions and thereby may play a role in stabilizing the A11 mode of alignment in keratin IF.

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MATERIALS AND METHODS

Expression and Purification of K5 and K14 Chains—Full-length human K5 and K14 cDNAs were assembled into a pET11a vector and expressed in bacteria as described (12). Several mutant forms of both chains were generated by use of the QuickChange site-directed mutagenesis kit (Stratagene) (Table II). DNA sequencing was performed to confirm the mutations. Following induction, inclusion bodies were re-

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‡ The abbreviations used are: IF, intermediate filament; DST, disulfosuccinimidyl tartrate; K, keratin, as in K5 (keratin 5); TOEP, tris(2-carboxyethyl)phosphine-HCl; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; GFP, green fluorescent protein.
covered, dissolved in SDS-PAGE buffer, and resolved in 3-mm-thick slab gels. The desired keratin bands were cut out, eluted into SDS gel buffer over night, and the solutions stored at 270 °C. Protein concentrations were determined by amino acid analysis following acid hydrolysis.

In Vitro IF Assembly—Equimolar mixtures of either a wild type and/or mutant K5 and K14 chain were made from the stored SDS gel buffer solutions. The SDS was removed by ion-pair extraction (13) and the pelleted acetone-wet proteins redissolved (0.05 or 0.5 mg/ml) in a buffer of 9.5M urea containing 50 mM Tris-HCl (pH 7.6), 5 mM Tris(2-carboxyethyl)phosphine-HCl (TCEP) (Pierce), and 1 mM EDTA. For electron microscopy studies, IF were assembled by 1-h dialyses through solutions of decreasing urea solutions of 4, 2, and 1M, and finally into assembly buffer of 10 mM Tris-HCl (pH 7.6), 1 mM EDTA and 5 mM TCEP (12). Final protein concentrations were 35–40 mg/ml, which is below the critical concentration \( C_0 \) for IF assembly (14), wherein mostly two-molecule assemblies formed, or 400 mg/ml for optimal IF assembly. Particles were examined by electron microscopy following negative staining with 0.2–0.7% uranyl acetate over holey carbon film grids. Lengths of IF were measured (15) in fields of $400 \, \mu m^2$. For IF assembly efficiency studies, protein mixtures in 9.5 M urea (40 mg/ml) were dialyzed directly into assembly buffer for 4 h. Solutions were then pelleted at 100,000 x g for 30 min in an Airfuge (Beckman Instruments). Yields of protein in pellet were estimated by measuring the absorbance at 276 nm of the supernatant.

Transfection Experiments with K14-Green Fluorescent Protein (GFP) Plasmids—A construct encoding GFP coupled at the 5' end of the full-length coding sequence of wild type K14 was a generous gift of Dr. R. D. Goldman (Northwestern University Medical School, Chicago, IL). Point mutations were made in the plasmid as described above. PtK2 (NBL-5) cells, epithelial-like rat kangaroo kidney cells, were obtained from ATCC (no. CCL-56). The cells were grown in 25-cm² tissue culture flasks and maintained in MEM (Eagle's minimal essential medium with nonessential amino acids, Earle's salts and reduced sodium bicarbonate at 0.85 g/liter) (Life Technologies, Inc.) with 10% fetal bovine serum. For cell passage, the cells were grown to near confluence, and the medium was aspirated, washed once with phosphate-buffered saline, and trypsinized for 20 s (0.25% trypsin; Life Technologies, Inc.). The trypsin solution was aspirated, and the cells

![Fig. 1. Distribution of charged residues in the K5 (upper row of each pair) and K14 (lower row) chains in the 1A, L1, 1B, L12, 2A, L2, and first part of 2B rod domain segments. Conserved charged positions are colored red (acids) or blue (basics). Non-conserved (black) or oppositely charged (green) positions are also shown. a–g denote the heptad position. Numbers on right denote the domain residue position number, not protein sequence residue number.](image1)

![Fig. 2. Electron microscopy of wild type and IF assembled from wild type and/or mutant K5/K14 chains. These and all data are summarized in Table III. A, wild type; B, K5 wild type and K14 1A Arg → Leu; C, K5 wild type and K14 1A Arg → Lys; D, K5 1A Lys → Arg and K14 wild type; E, K5 wild type and K14 1A Lys → Met; F, K5 wild type and K14 2A Asp → Ala; G, K5 L2 Arg → Leu and K14 wild type; H, K5 L2 Glu → Ala/Glu → Ala and K14 wild type. Scale bar, 200 nm.](image2)
were left at room temperature for 3 min. Five milliliters of medium were pipetted over the cells to dislodge them from the flask, and transferred to a 15-ml conical tube. Following 5 min of 1000 rpm centrifugation to pellet the cells, the medium was aspirated, and the cells were resuspended in 2 ml of medium and counted.

For direct immunofluorescence studies, $3 \times 10^5$ cells/ml were plated in 35-mm sterile tissue culture dishes, each containing a glass coverslip. After 24 h, the cells were transfected with 1 mg of plasmid DNA and 3 mg of Lipofectin as described by the manufacturer (Life Technologies, Inc.). After 4 h, the mix was aspirated and 1 ml of 15% glycerol in Keratinocyte-SFM (Life Technologies, Inc.) was applied for 3.5 min. The glycerol solution was replaced with 2 ml of fresh medium and the cells incubated at 37 °C with 5% CO2 for at least 24 h. The coverslips were washed in phosphate-buffered saline and mounted onto glass slides with Gel/Mount (Biomeda Corp.). Intracellular localization of GFP fusion proteins was determined by direct fluorescent microscopy.

Protein Chemistry Procedures—To examine molecular stabilities, equimolar mixtures of the desired K5/K14 chains (~40 µg/ml) were equilibrated by 2-h dialyses into urea solutions of the desired concentration in a buffer of 10 mM triethanolamine (pH 8.0). The proteins were cross-linked with 25 mM disulfosuccinimidyl tartrate (DST) for 1 h at 23 °C, and terminated with 0.1 M NH4HCO3 (final concentration) (16). Although significant random cross-linking also occurs, these conditions were used because the near quantitative modification of all lysines allows for less diffuse bands on 3.75–7.5% gradient PAGE gels.

To assess molecular alignments in the A11 and A22 modes, cross-linking with DST was performed using 0.4 mM reagent as described before (8, 9). We used wild type and mutant proteins that had been equilibrated into assembly buffer at about 40 µg/ml for 1 h. In this case, <10% of the lysine residues were chemically modified, except for several aligned residues that formed cross-links with yields of up to about 0.3 mol/mol. Following cleavage with CNBr and trypsin digestion, peptides were resolved by HPLC as before, except that a non-linear gradient over a 120-min time period was used. The positions of elution of the peptides cross-linked by DST corresponding to the A11 and A22 molecular alignment modes were similar to those published previously (9), although many were confirmed by sequencing for five Edman degradation cycles on a Porton LF-3000 sequencer. Semiquantitative estimates of molar yields of each were made based on peak heights of the integrated HPLC profiles.

RESULTS AND DISCUSSION

In this paper we have made a systematic analysis of those charged residues that, based on current structural information, are located in rod domain positions that could influence the specificity and stability of the A11 alignment mode of a pair of antiparallel heterodimer molecules in K5/K14 IF. These encompass the segments 1A, 1B, 2A, and beginning of the 2B, as

![Figure 3](http://www.jbc.org/)

**FIG. 3.** Direct immunofluorescence of wild type and mutant GFP-K14 constructs transfected into PtK2 cells. These and all other data are summarized in Table III. A, wild type; B, 1A Arg10 → Leu; C, 1A Lys11 → Met; D, 1A Lys31 → Met; E, 1B Glu10 → Ala; F, 1B Glu14 → Ala; G, 1B Glu13 → Ala; H, 2B Glu196 → Ala.

![Figure 4](http://www.jbc.org/)

**FIG. 4.** Stabilities of dimer (one-molecule) and tetramer (two-molecule) assemblies of wild type and/or mutant chains in concentrated urea solutions (as shown) following cross-linking. These and all other data are summarized in Table IV. The compositions of the assembly reactions are as shown. T, D, and M, respectively, mark the position of migration of the tetramer (two-molecule), dimer (one-molecule), and single-chain species.
well as the linkers L1, L12, and L2. We found that 41 charged positions have been conserved in the type II keratin 5 (K5) (Fig. 1, upper row) and type I K14 (Fig. 1, lower row) chains. Based on extant ideas (2–4), we have hypothesized in this study that some of these may influence molecular alignment stabilities. Indeed, using the known quantitative estimate of molecular spacing of the A11 alignment mode for keratin IF (about 211 residues) (9), we document in Table I that most of the 41 conserved charged residue positions lie opposite to each other and so are well sited to theoretically form stabilizing ionic salt bond pairs and/or H-bonds. Nevertheless, we discharged all conserved charged residue positions (i.e. mutated them to a non-charged residue) (all mutations are listed in Table II). In addition, there are 59 residue positions in this set that are not conserved between the K5 and K14 chains, and 4 others that are oppositely charged; several of these are also theoretically good candidates to form stabilizing salt bonds (Table I). Some of these residue positions were discharged as well. We then examined the facility with which equimolar mixtures of one mutant and one wild type chain could assemble into one- and two-molecule oligomers, as well as IF in vitro and in vivo.

Assembly of IF in Vitro and in Vivo—The initial criterion of assembly competence was formation of pelletable IF particles by use of a sedimentation assay in the Airfuge. Experience has shown that particles must be $750 \text{ kDa}$ in size in order to pellet with high efficiency. This corresponds to an oligomer of as many as 16 chains (8 molecules), i.e. it consists of a full-length half-width entity characteristic of an early stage of IF assembly (17). In almost all cases, however, we found empirically that assembly of mixtures of mutant and/or wild type chains either resulted in macroscopic IF (0.5 mm long), which were readily pelletable in the Airfuge and clearly visible by electron microscopy after negative staining, or no large IF particles were formed at all (0.1 mm long and 4 nm wide), which did not pellet in the Airfuge and required examination over holey carbon film grids to be visible.

Sixty-six combinations of K5/14 chains were examined in in vitro assays (Fig. 2, Table II). These included every case of a conserved charged residue position in the K5 and K14 chains documented in Fig. 1, as well as several other positions that had not been conserved. Almost all formed pelletable particles 2 L. N. Marekov, D. A. D. Parry, and P. M. Steinert, unpublished observations.

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**Fig. 6.** A model of two heterodimer keratin molecules (the two chain types are shown in white and green) aligned in the A11 mode using the statistically averaged displacement values determined from earlier cross-linking studies (2, 3, 10). This displays the close proximity of the two conserved and Arg/Lys10 residues of one molecule (blue lines, large blue dots) with the conserved acidic residues Glu4 and Glu6 in the L2 linker (red lines, large red dots) of the other molecule. In addition, large dots delineate the possible interactions between 1A Lys10 and 2A Asp1. Smallers dots delineate possible interactions involving 2A Asp3 and L2 Glu7. We hypothesize that these may form several intermolecular salt and/or H-bonds and thereby contribute essential specificity and stability to the A11 alignment mode. The segments of the molecules are marked.
in high yield and appeared as native-type IF >1 μm in length. However, several combinations did not, including those positions in the 1A rod domain segment (K14 Asp → Ala, Arg→ Leu, K5 Lys → Met, K14 Lys → Glu or Lys → Met), three positions in the L2 linker (several combinations of K5 and/or K14 in which positions 4, 6, and 7 (K14 chain only) of L2 had been discharged), and three positions in the 2B rod domain segment (residues 100, 104, and 106). In addition, the 2A rod domain residue Asp → Ala (K5) produced particles that only partially pelleted in the Airfuge and visibly were only 0.1–0.3 μm in length (Table III).

In a related second set of experiments, nine of these mutations were introduced into the GFP-K14 construct and their propensities for assembly into keratin IF in vitro were examined after transfection into PtK2 cells (Fig. 3). These cells express predominantly the K6, K7, K16, and K17 keratin chains but have been shown previously to accommodate incorporation of transfected wild type or mutant K14 chains (19). Additionally, the efficacy of incorporation of transfected GFP-K14 constructs into cultured cells to explore keratin IF cytoskeletons is now established (20). Four mutants (1A Arg→ Ala (Fig. 3A), 1A Lys → Met (Fig. 3D), K5 Lys → Ala (Fig. 3G), and 2B Lys → Ala (Fig. 3H)), resulted in severely disrupted cytoskeletons in which most of the keratin IF had withdrawn to a perinuclear location, and there were bright spots of unassembled GFP-labeled protein. In five other cases, the keratin IF cytoskeletons were either unchanged (1A Lys → Met (Fig. 3C), 1B Lys → Ile (data not shown)), or mildly abnormal due to some apparent clumping and/or elongation of the keratin IF (1A Glu → Ala (data not shown), 1B Glu → Ala (Fig. 3E), and 1B Glu → Ala (Fig. 3F)).

Some of these data were expected and thus serve as controls. The 1A positions 9 and 10 have been shown to be of importance in this study.

Cross-linking Studies with DST in Urea Solutions to Assess One- and Two-molecule Stabilities—The second criterion of assembly competence used in this study was the formation of stable one- and two-molecule assemblies. We have previously established (16) a method to assess the stabilities of single coiled-coil molecules and pairs of them by use of a graduated urea concentration titration assay coupled with cross-linking by DST. At protein concentrations below the critical concentration for IF assembly (~40 μg/ml) in assembly buffer in the absence of urea, the K5 and K14 chains form mostly two-molecule (and traces of one-, three- and four-molecule) oligomers (8, 9). These dissociate into single molecules at about 6.5 μM urea (approximate concentration of half loss), and then the molecules dissociate to individual chains by about 9.5 μM urea, as reported earlier by Wawersik et al. (22) for K5/14 keratin IF and for vimentin and α-internexin (16) (see Fig. 4A).

Mutants representing every single conserved charged residue position in either the K5 or K14 chain (from Fig. 1), and some nonconserved ones, were tested in this assay. Several observations are apparent (Table IV). First, for only the 2B rod domain positions 100, 104, and 106 were both the two-molecule and single-molecule entities unstable in even 1 μM urea. This is expected from our earlier data, as these residues participate in the formation of a stabilizing coiled-coil trigger motif for IF (11). Second, in all other cases, the one-molecule species was essentially as stable as the wild type. However, third, there were several conserved charged residue positions that resulted in significantly destabilized two-molecule entities (~4 μM urea), including 1A positions 9 and 10; 2A position 1; and L2 positions 4, 6, and 7. Finally, in a few other positions, the two-molecule entity was somewhat less stable (~5 μM): 1A position 31 and 2A position 3.

Taken together with the in vivo and in vitro IF assembly experiments, these data reveal that 1A positions 9, 10, and 31; 2A positions 1 and 3; and linker L2 positions 4, 6, and 7 are important for the stability of the two-molecule entity of K5/14. The Arg Residue of the 1A Rod Domain Segment and the Glu and Glu Residues of the L2 Linker Are Especially Required for the Stability of the Alignment Mode of Two Molecules—We have documented that there are three possible modes of alignment of a pair antiparallel molecules to form the

### Table I

| Chain/location | First position | Potential partner | Axis position |
|----------------|---------------|-------------------|--------------|
| K14 1B-56 Asp | 107.27        | K5 1B-42 Lys      | 107.38       |
| K14 1B-38 Asp | 89.27         | K14 1B-40 Arg     | 109.38       |
| 1B-37 Asp     | 88.27         | K5 1B-62 Lys      | 87.38        |
| K14 1B-33 Arg | 84.27         | K14 1B-61 Lys     | 83.38        |
| K5 1B-28 Glu  | 79.27         | K14 1B-65 Asp     | 84.38        |
| K14 1B-12 Arg | 63.27         | K14 1B-71 Arg     | 73.38        |
| K14 1A-31 Lys | 31.00*        | K14 1B-83 Lys     | 66.38        |
| K14 1A-22 Glu | 22.00         | K14 2A-01 Asp     | 33.44*       |
| K14 1A-19 Arg | 19.00         | K5 2A-03 Asp      | 31.44*       |
| K5 1A-16 Asp  | 16.00         | K14 2A-10 Arg     | 24.44        |
| K14 1A-10 Arg | 10.00*        | K14 2A-14 Lys     | 20.44        |
| K5 1A-10 Lys  | 10.00*        | K14 2A-19 Lys     | 15.44        |
| K5 2A-03 Arg  | 11.01*        | K5 L2-02 Arg      | 14.18        |
| K14 1B-33 Asp | 11.01*        | K14 L2-07 Glu     | 11.01*       |
| K14 1B-61 Glu | 11.65*        | K14/5 1B-06 Glu   | 11.65*       |
| K14 1B-71 Lys | 12.91*        | K14/5 1B-04 Asp   | 12.91*       |
| K14 1B-84 Glu | 6.38*         | K14 2B-04 Lys     | 6.38*        |
| K5 1B-83 Lys  | 3.38          | K14 2B-07 Glu     | 3.38         |

* Axial positions are measured in terms of h, which corresponds to a 0.1485-nm rise of each residue in a coiled coil conformation. This list documents the oppositely charged residues that are located within ±3 residues of each other (and thus theoretically could form a salt bond) when aligned using the known parameters of A11 mode added for keratin IF. Asterisks identify those residue positions/pairs which were confirmed to be of importance in this study.
two-molecule oligomer, known as $\Lambda_{11}$, $\Lambda_{22}$, and $\Lambda_{12}$. In our hands, the $\Lambda_{12}$ mode exists only at high pH values and is not assembly-competitive (14). However, a variety of chromatographic, ultracentrifugation, electron microscopic, solution bi-refringence, and cross-linking data have documented that two-molecule oligomer of a variety of mammalian IF exist in assembly-competent solutions. As 60–70 nm-long particles in which the two molecules are aligned in the $\Lambda_{11}$ and/or $\Lambda_{12}$ mode. Our previous cross-linking experiments with keratin filaments have shown that the two must co-exist in solution, since we have been able to recover DST cross-linked peptides arising from links between antiparallel molecules aligned in both modes (8–10). Therefore,
we reasoned in the present experiments that the destabilization of the two-molecule oligomer in the several mutations identified above should be due to loss of one or both of these alignment modes. To check this, we performed additional larger scale cross-linking experiments with 0.4 mM DST. The proteins were then cleaved with CNBr and trypsin, and the resulting peptides were resolved by HPLC (Fig. 5), but using a broader and flatter gradient extending over 120 min versus 70 min previously (8). We found six common peaks in the one- and two-molecule species of wild type K5/14 arising from intramo-

| Mixture | In vitro assembly | GFP-K14 in vivo assembly |
|---------|------------------|--------------------------|
|         | Figure | Description | Figure | Description |
| 1A      | Wild type | Wild type | 2A | Normal | 10 μm | 3A | Normal |
| Arg1 → Leu | Wild type | Normal | 5 μm | None | Disrupted, spots |
| Wild type | Asp9 → Ala | None | 2B | None | Disrupted, spots |
| Wild type | Arg10 → Leu | 2C | Shorter | 2 μm | Disrupted, spots |
| Lys11 → Met | Wild type | None | 2D | Shorter | 2 μm | Abnormal |
| Lys11 → Arg | Wild type | Shorter | 1 μm | Shorter | 2 μm |
| Asp18 → Ala | Wild type | Shorter | 1 μm | Shorter | 2 μm |
| Wild type | Lys17 → Met | Normal | 5 μm | Normal | 2 μm |
| Wild type | Lys19 → Leu | Shorter | 2 μm | Shorter | 2 μm |
| Wild type | Glu22 → Ala | Short | 1 μm | Shorter | 2 μm |
| Wild type | Glu29 → Ala | Short | 1 μm | Shorter | 2 μm |
| Wild type | Lys31 → Glu | None | None | 2E | None | Disrupted, spots |
| Wild type | Lys31 → Met | Shorter | 2 μm | Normal | 5 μm |
| Wild type | Glu44 → Ala | Shorter | 2 μm | Normal | 5 μm |
| Lys49 → Met | Wild type | Normal | 5 μm | Normal | 5 μm |
| Wild type | Asp36 → Ala | Normal | 5 μm | Abnormal |
| Lys61 → Met | Wild type | Shorter | 2 μm | Normal |
| Wild type | Asp65 → Ala | Shorter | 2 μm | Shorter | 2 μm |
| Lys17 → Ile | Wild type | Shorter | 2 μm | Shorter | 2 μm |
| Wild type | Arg71 → Ile | Normal | 5 μm | Normal |
| Wild type | Asp73 → Ala | Shorter | 2 μm | Shorter | 2 μm |
| Wild type | Glu75 → Ala | Normal | 5 μm | Normal |
| Wild type | Glu79 → Ala | Normal | 5 μm | Normal |
| Wild type | Lys82 → Glu | Shorter | 2 μm | Shorter | 2 μm |
| Asp36 → Ala | Wild type | Shorter | 2 μm | Shorter | 2 μm |
| Wild type | Glu84 → Ala | Shorter | 1 μm | Shorter | 2 μm |
| Lys89 → Met | Wild type | Shorter | 2 μm | Normal |
| Wild type | Glu95 → Ala | Shorter | 2 μm | Abnormal |
| 2A | Asp1 → Ala | Wild type | Very short | <1 μm | Normal |
| Asp9 → Ala | Wild type | Short | 1 μm | Normal |
| Wild type | Glu18 → Ala | Short | 1 μm | Normal |
| Wild type | Arg10 → Leu | Short | 1 μm | Normal |
| Glu14 → Ala | Wild type | Normal | 5 μm | Normal |
| Wild type | Lys15 → Met | Shorter | 2 μm | Normal |
| Wild type | Lys19 → Met | Shorter | 2 μm | Normal |
| L2 | Arg2 → Leu | Wild type | Short | 1 μm | Normal |
| Glu4 → Ala | Wild type | None | None | None | Disrupted, spots |
| Wild type | Asp9 → Ala | None | None | None | Disrupted, spots |
| Wild type | Glu6 → Ala | None | None | None | Disrupted, spots |
| Wild type | Glu7 → Ala | None | None | None | Disrupted, spots |
| Wild type | Asp9 → Ala/Glu6 → Ala | Wild type | None | None | Disrupted, spots |
| Glu14 → Ala | Wild type | None | None | None | Disrupted, spots |
| Wild type | Asp9 → Ala/Glu7 → Ala | None | None | None | Disrupted, spots |
| Arg2 → Leu | Asp9 → Ala | None | None | None | Disrupted, spots |
| Arg2 → Leu | Glu6 → Ala | None | None | None | Disrupted, spots |
| Glu4 → Ala | Asp9 → Ala | None | None | None | Disrupted, spots |
| Glu4 → Ala | Glu7 → Ala | None | None | None | Disrupted, spots |
| 2B | Wild type | Lys4 → Met | Normal | Normal | Normal |
| Wild type | Glu6 → Ala | Normal | Normal | Normal |
| Wild type | Glu7 → Ala | Normal | Normal | Normal |
| Wild type | Glu17 → Ala | Normal | Normal | Normal |
| Lys190 → Met | Wild type | None | None | None | Disrupted, spots |
| Asp99 → Ala | Wild type | None | None | None | Disrupted, spots |
| Wild type | Glu106 → Ala | None | None | None | Disrupted, spots |
### Table IV

**Molecular stabilities of K5/K14 wild type and mutant chain assemblies in concentrated urea solutions**

| Composition | [Urea] of half-disassembly | Figure |
|-------------|---------------------------|--------|
|             | Tetramer                  | Dimer  |          |
| K5          | K14                       |        |          |
| Wild type   | Wild type                 | 6.5    | 9        | 4A      |
| Arg13 → Leu| Wild type                 | 6      | >8       |         |
| Wild type   | Asp9 → Ala                | 4      | >8       |         |
| Wild type   | Arg10 → Leu               | 3.5    | 8        | 4B      |
| Wild type   | Arg10 → Lys               | 5.5    | >8       |         |
| Wild type   | Arg10 → Gin               | 4.5    | 8        |         |
| Lys10 → Met| Wild type                 | 3.5    | 8        |         |
| Lys10 → Arg| Wild type                 | 5.5    | >8       | 4C      |
| Asp16 → Ala| Wild type                 | >5.5   | 8        |         |
| Wild type   | Lys17 → Met               | 6      | 8        |         |
| Wild type   | Arg19 → Leu               | >5.5   | 8        |         |
| Wild type   | Glu22 → Ala               | 5.5    | >7       |         |
| Wild type   | Glu29 → Ala               | 5.5    | 8        |         |
| Wild type   | Lys31 → Glu               | <5     | 8        |         |
| Wild type   | Lys31 → Met               | <5     | 9        | 4D      |
| 1B          |                           |        |          |
| Wild type   | Arg12 → Met               | 6      | 8        |         |
| Wild type   | Asp21 → Ala               | 6      | >8       |         |
| Glu20 → Ala| Wild type                 | 6      | 8        |         |
| Wild type   | Arg33 → Leu               | >6     | 8        |         |
| Wild type   | Asp37 → Ala               | 6      | 8        |         |
| Wild type   | Asp38 → Ala               | 6      | 8        |         |
| Wild type   | Arg39 → Leu               | 6      | 8        |         |
| Lys42 → Met| Wild type                 | 6      | 8        |         |
| Lys39 → Met| Wild type                 | >6     | 8        |         |
| Glu34 → Ala| Wild type                 | >6     | >8       |         |
| Wild type   | Asp36 → Ala               | 6      | >8       |         |
| Lys30 → Met| Wild type                 | 6      | 8        |         |
| Lys32 → Met| Wild type                 | 6      | 8        |         |
| Wild type   | Asp35 → Ala               | 6      | >7.5     |         |
| Lys71 → Ile| Wild type                 | 5.5    | >8       |         |
| Wild type   | Arg71 → Ile               | 5.5    | 8        |         |
| Wild type   | Asp73 → Ala               | 5.5    | 8        |         |
| Wild type   | Glu75 → Ala               | 6      | 8        |         |
| Wild type   | Asp79 → Ala               | 6      | 8        |         |
| Wild type   | Lys82 → Glu               | >5.5   | 8        |         |
| Asp83 → Ala| Wild type                 | >5.5   | >7.5     |         |
| Wild type   | Glu34 → Ala               | >5.5   | >8       |         |
| Lys89 → Met| Wild type                 | >5.5   | 8        |         |
| Wild type   | Glu95 → Ala               | >5.5   | 8        |         |
| 2A          |                           |        |          |
| Wild type   | Asp9 → Ala                | 4      | >7.5     | 4E      |
| Asp9 → Ala | Wild type                 | 4.5    | 8        |         |
| Wild type   | Glu92 → Ala               | >5.5   | 8        |         |
| Wild type   | Arg92 → Leu               | >5.5   | >8       |         |
| Glu14 → Ala| Wild type                 | 6      | 8        |         |
| Wild type   | Lys15 → Met               | 6      | 8        |         |
| Wild type   | Lys19 → Met               | >5.5   | 8        |         |
| L2          |                           |        |          |
| Arg6 → Leu | Wild type                 | 5.5    | >8       | 4F      |
| Glu4 → Ala | Wild type                 | 4      | 8        |         |
| Wild type   | Asp6 → Ala                | 4      | 8        |         |
| Glu6 → Ala | Wild type                 | 4      | 8        |         |
| Wild type   | Glu1 → Ala                | 4      | 8        |         |
| Wild type   | Glu1 → Ala                | >4     | 8        |         |
| Wild type   | Asp6 → Ala/Glu6 → Ala     | <4     | 8        | 4G      |
| Wild type   | Glu1 → Ala/Glu6 → Ala     | <4     | 8        |         |
| Wild type   | Asp6 → Ala/Glu7 → Ala     | <4     | >7.5     |         |
| Arg6 → Leu | Asp6 → Ala                | 4      | >7       |         |
| Arg6 → Leu | Glu1 → Ala                | <4     | >7       |         |
| Arg6 → Leu | Glu1 → Ala                | 4      | 8        |         |
| Glu6 → Ala | Asp6 → Ala                | <4     | >7       |         |
| Glu6 → Ala | Glu1 → Ala                | <4     | >7       |         |
| Glu6 → Ala | Glu1 → Ala                | <4     | >7       |         |
| 2B          |                           |        |          |
| Wild type   | Lys10 → Met               | >5.5   | >8       |         |
| Wild type   | Glu6 → Ala                | 6      | 8        |         |
| Wild type   | Glu6 → Ala                | 6      | >8       |         |
| Wild type   | Glu17 → Ala               | >6     | >8       |         |
| Wild type   | Lys33 → Ile               | <1     | >8       |         |
| Lys100 → Met| Wild type                 | <1     | 1        |         |
| Asp104 → Ala| Wild type                 | <1     | 1        |         |
| Wild type   | Glu106 → Ala              | <1     | 1        | 4H      |
Stability of the $A_{11}$ Alignment in Keratin Filaments

**Table V**
Yields of peptides in the $A_{11}$ and $A_{22}$ two-molecule alignments (mol/mol)

| Cross-link | K5: wt$^a$ | wt | wt | wt | L2 E06A | L2 E06A | L2 E06A | wt |
|------------|-----------|----|----|----|---------|---------|---------|----|
| A$_{11}$ cross-links | K14: wt | 0.07 | 0.00 | 0.10 | 0.02 | 0.02 | 0.00 | 0.00 | 0.08 |
| K14: 2A 19 | 0.25 | 0.01 | 0.22 | 0.05 | 0.03 | 0.00 | 0.00 | 0.11 |
| K14: 1B 40 | 0.06 | 0.00 | 0.05 | 0.01 | 0.00 | 0.00 | 0.00 | 0.04 |
| K14: 1B 61 | 0.33 | 0.02 | 0.35 | 0.05 | 0.03 | 0.00 | 0.00 | 0.13 |
| K14: 1B 61 | 0.10 | 0.00 | 0.09 | 0.01 | 0.01 | 0.00 | 0.00 | 0.03 |
| K14: 1B 61 | 0.09 | 0.00 | 0.06 | 0.01 | 0.01 | 0.00 | 0.00 | 0.02 |
| K14: 1B 61 | 0.03 | 0.00 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 |
| K14: 1B 61 | 0.17 | 0.00 | 0.18 | 0.02 | 0.02 | 0.00 | 0.00 | 0.05 |
| K14: 1B 61 | 0.25 | 0.01 | 0.20 | 0.01 | 0.04 | 0.00 | 0.00 | 0.10 |
| K14: 1B 61 | 0.03 | 0.00 | 0.01 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 |
| K14: 1B 61 | 0.10 | 0.15 | 0.08 | 0.11 | 0.10 | 0.09 | 0.10 | 0.00 |
| K14: 1B 61 | 0.05 | 0.09 | 0.05 | 0.06 | 0.06 | 0.06 | 0.06 | 0.05 |
| K14: 1B 61 | 0.10 | 0.12 | 0.09 | 0.12 | 0.11 | 0.10 | 0.11 | 0.00 |
| K14: 1B 61 | 0.08 | 0.12 | 0.10 | 0.10 | 0.14 | 0.13 | 0.11 | 0.00 |
| K14: 1B 61 | 0.14 | 0.16 | 0.18 | 0.15 | 0.14 | 0.15 | 0.18 | 0.00 |

$^a$ wt, wild type.

Substitutions in Keratinopathy Diseases—Thus, we have presented three sets of data, which document that certain residues along the keratin IF chains are especially important for: successful IF formation *in vitro* and *in vivo*; the stability of the two-molecule hierarchical stage of IF assembly; and, in particular, for specifying and stabilizing the $A_{11}$ mode of alignment of two antiparallel molecules. Indeed, several of the residues identified here correspond to residue pairs documented in Table I that are theoretically good candidates to form stabilizing ionic salt bonds. Based on the known alignment parameters of two antiparallel molecules in the $A_{11}$ mode, these residues are likely to lie very close to each other in the $A_{11}$ mode (Fig. 6).

Thus, the conserved $A_{11}$ position of the 1A rod domain segment is closely adjacent to the conserved set of two (and in type I IF chains, three) acidic residues in positions 4, 6, and 7 in the linker L2. Notably, discharging of any one of these residues severely compromised the $A_{11}$ alignment of the two-molecule hierarchical stage of IF structure. Asp$^9$ (often an isosteric Asn in many IF chains) is likewise adjacent to these residues in L2. In addition, we note from Fig. 6 that the conserved 1A residue Lys$^{31}$ lies near the conserved 2A residue Asp$^9$ (K5 chain only); likewise, discharging of these residues resulted in impaired stability of the $A_{11}$ alignment mode.

The simplest explanation of these data is that the key residues identified in this study interact to afford essential stability. One possibility is that this stability is provided by the formation of a complex intermolecular network of salt bonds and/or H-bonds. However, we cannot formally exclude the possibility that head and/or tail domain sequences also cooperate in these stabilizing phenomena. In addition, it is to be expected

Model for the Stabilization of the $A_{11}$ Alignment Mode in Keratin IF: A Molecular Explanation for the Role of Arg$^{10}$

In the wild type two-molecule oligomer, there were an additional 15 peaks due to intermolecular links, of which 5 could be assigned to linkages between molecules aligned in the $A_{22}$ mode (Fig. 5C, closed circles), and 10 to linkages denoting the $A_{11}$ alignment mode (Fig. 5C, open circles). Semiquantitative data on the amounts of each were determined on the basis of peak areas (all data summarized in Table V). These experiments were repeated for seven mutant mixtures. As found previously (11), the Glu$^{106}$ → Ala substitution in the 2B rod domain segment resulted in loss of the $A_{22}$ alignment mode, and resultant substantial loss of the $A_{11}$ mode. However, the 1A Arg$^{10}$ → Leu (Fig. 5D) and Lys$^{31}$ → Met, and 2B Glu$^6$ → Ala single (Fig. 5E) or Glu$^4$ → Ala/Glu$^6$ → Ala double substitutions resulted in almost complete loss of the $A_{11}$ mode. Further, the yields of the cross-links denoting to the $A_{22}$ mode were generally increased over the wild type amounts (Table V). These data confirm that the $A_{11}$ and $A_{22}$ modes of molecular alignment in fact exist in equilibrium in solution and suggest that loss of the former by destabilization results in a net reduction of the stability of all tetramers, together with an accumulation of molecules into the latter. The Arg$^{10}$ → Lys substitution in 1A retained near wild type yields of both modes, consistent with the data of Tables III and IV. In summary, these data document that discharging of Arg$^{10}$ of 1A, or one or both of the acidic residues Glu$^4$ and Glu$^6$ of the L2 linker singly or together, results in loss of the $A_{11}$ mode of alignment. Discharging of Lys$^{31}$ in 1A results in substantial loss of the $A_{11}$ mode.
that many other charged residues, in addition to the key ones identified here, may also contribute in important ways to the alignment of the A_{11} mode. It is also possible that these residues may participate in higher orders of IF structure, in particular the lateral association of molecules in the A_{12} alignment mode, and elongation of molecules by overlapping of the A_{CN} alignment mode. The availability of the complete atomic structure of a single IF molecule should provide the opportunity to further explore these possibilities in model building studies. Finally, it is interesting to note that the key potential interactions identified here do not involve the 1B segment, which corresponds to the central region of the A_{11} overlap. Instead, both ends appear to be crucial in making favorable intermolecular interactions. Nevertheless, we speculate that apolar interactions between residues in the antiparallel 1B segments could also play a role in stabilizing the A_{11} alignment mode.

Interestingly, substitution of the Arg^{10} residue in the 1A rod domain segment of especially type I keratins often results in a very serious phenotype in a variety of keratinopathy diseases (recently reviewed in Ref. 18). The molecular basis of the consequence of this substitution on keratin IF structure has not heretofore been determined, although one report (23) suggested the problem occurred at a structural hierarchical level above the stability of a single molecule. Our present data indicate in a straightforward way that this substitution causes a serious problem at the level of the two molecule stage of IF assembly, in particular by destabilizing the A_{11} alignment mode.

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