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Interaction of Collagen Molecules from the Aspect of Fibril Formation: Acid-Soluble, Alkali-Treated, and MMP1-Digested Fragments of Type I Collagen[1]

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Collagen type I extracted with acid or digested with pepsin forms fibrils under physiological conditions, but this ability is lost when the collagen is treated with alkaline solution or digested with matrix metalloproteinase 1 (MMP1). When acid-soluble collagen was incubated with alkali-treated collagen, the fibril formation of acid-soluble collagen was inhibited. At 37°C, at which alkali-treated collagen is denatured, the lag time was prolonged but the growth rate of fibrils was not affected. At 30°C, at which the triple helical conformation of alkali-treated collagen is retained, the lag time was prolonged and the growth rate reduced. Heat-denatured alkali-treated collagen and MMP1-digested fragments have no inhibitory effect on the fibril formation of acid-soluble collagen. This means that the triple helical conformation and the molecular length are important factors in the interaction of collagen molecules and that alkali-treated collagen acts as a competitive inhibitor for fibril formation of collagen. We found that alkali-treated collagen and MMP1-digested fragments form fibrils that lack the D periodic banding pattern and twisted morphology under acidic conditions at the appropriate ionic strength. We also calculated the relative strengths of hydrophobic and electrostatic interactions between collagen molecules. When the hydrophobic interaction between linear collagen molecules was considered, we found a pattern of periodic maximization of the interactive force including the D period. On the other hand, the electrostatic interaction did not show the periodic pattern, but the overall interaction score affected fibril formation.

Key words: alkali-treatment, collagen, fibril formation, hydrophobic interaction, MMP.

Fibrillar collagen is the major protein in connective tissue; its fibrils provide mechanical strength to tissues and form a favorable matrix for cell attachment *in vivo* (*1-3*). The mechanism of collagen fibril formation has long been studied. Most of these studies were performed using type I collagen extracted from tissue by acid or pepsin treatment. Fibril formation of type I collagen is initiated by neutralizing and warming the collagen solution (*4, 5*). A system for studying *de novo* fibril formation of type I collagen has also been developed in which fibril formation is initiated by cutting the C-propeptide of procollagen using a specific enzyme (*6*). Using this system, pointed tips in the C- to N-terminal direction of the growing fibrils were found (*5, 7*). The interacting force of collagen molecules for fibril formation has also been studied. A charged amino acid plays an important role in the formation of SLS (segment-long-spacing crystallites) fibrils (*8, 9*), and the hydrophobic interactions have been suggested to be important in stabilizing the assembled collagen fibril with a D period (*8, 10*).

In another method for investigating collagen fibril formation, inhibiting or accelerating agents have been used. Glucose and urea are known to be inhibitors of fibril formation and low amounts of SDS stimulate fibril formation (*11, 12*). Concerning the stabilizing effect of N- and C-telopeptides on collagen fibrils, the following two independent studies have been reported: one shows the inhibitory effect of pepsin-solubilized collagen on acid-solubilized collagen fibril formation (*13*), and the other the inhibitory effect of synthetic peptide coding telopeptides on fibril formation (*14*). Chemical modifications of collagen molecules also alter the features of fibril formation. Methylation of the carboxylic group with methanolic hydrochloric acid (*15*), deamination of Asn and Gln residues with sodium hydroxide (*15, 16*), and succinylation of the ε-amino groups with succinyl anhydride (*15*) deprive collagen of fibril forming activity at neutral pH.

In this report, we examine the effect of alkali-treated collagen and collagen fragments digested with MMP1 on

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Abbreviations: ASC, acid-soluble collagen; CD, circular dichroism; h-d, heat denatured; MMP, matrix metalloproteinase; PBS, phosphate buffered saline.

the fibril formation of acid-soluble collagen at 30°C and 37°C (RESULTS I). The results show that alkali-treated collagen has an inhibitory effect on fibril formation, while MMP1-digested collagen fragments have little effect. Next we examined the fibril forming activity of alkali-treated collagen and MMP1-digested fragments under various conditions (RESULTS II) and found that these collagen preparations form fibrils under acidic conditions. In "RESULTS III," we calculated the forces of interaction between the triple helical region of collagen molecules considering the hydrophobic and electrostatic interactions, and glycosylations of the hydroxylysine residues.

MATERIALS AND METHODS

Preparation of Collagen—Acid-soluble collagen was prepared from steer hide. Briefly, crude collagen was extracted from steer hide with 0.5 M acetic acid and purified by salt fractionation and isoelectric precipitation. The precipitate was dissolved in 5 mM acetic acid. Salt fractionation and isoelectric precipitation were repeated twice at 4°C. The final concentration of the collagen solution was adjusted to 3.0 mg/ml.

Alkali-treated collagen was prepared as described before (*16*). In brief, acid-soluble collagen was treated with a 3.0% NaOH (w/v)-1.9% monomethylamine (w/v) solution for 14 days at 18°C. The solution was dialyzed sequentially against 0.5 M and 5 mM acetic acid and lyophilized. The denaturation temperature of acid-soluble collagen and alkali-treated collagen in PBS (phosphate buffered saline) as measured by CD (circular dichroism) was 40.4 and 34.6°C respectively (*16*).

Preparation of Collagen Fragments Digested with MMP1—Acid-soluble collagen was digested with human MMP1 (Yagai) at 20°C. The digestion was stopped by dialyzing the mixture against 5 mM acetic acid. The rate of digestion was estimated by densitometry comparing the digested fragments (TCa and TCb) with the undigested α chain by SDS-PAGE. We confirmed that more than 95% of collagen is digested to specific fragments. These fragments retain the collagen-specific triple helical conformation as measured by CD (circular dichroism). The denaturation temperature of the larger fragment (TCa) in PBS is 35.5°C.

Collagen Fibril Formation at Neutral pH—The collagen solution was neutralized in a disposable polystyrene cuvette (Kartell) to measure the absorbance by adding phosphate-buffered saline (×3 concentrated) with gentle shaking to make a final volume of 3 ml on ice. The neutralized collagen was incubated at 37 or 30°C, and the turbidity at 530 or 520 nm was measured every 2 min. Usually the process of collagen fibril formation comprises two phases, nucleation and growth, as in the case of crystallization or polymerization of biological molecules during the formation of an organized aggregate. To describe these phases, we used the extrapolated line of the slope at the mid-point of the turbidity crossing the abscissa on the time scale as the nucleation (lag) time, and the slope at the mid-point as the maximum growth rate. We describe T_0 for nucleation time and G_{max} for maximum growth rate. The turbidity of the collagen fibrils is thought to parallel to the concentration when the conditions are the same (*11*); therefore, the growth rate is expressed as the increase in collagen (μg) incorporated into the fibril per min in 1 ml of solution.

Collagen Fibril Formation at Acidic pH—To survey the fibril-forming ability of collagen over a wide range of pH or salt concentration, McIlvaine buffer (mixture of 0.1 M Na_2HPO_4 and 0.05 M citrate solution) was used. The collagen solution was mixed with McIlvaine buffer (pH 4.5 and 7.5) in the presence of NaCl (final concentration adjusted to 0.15 or 0.3 M). After warming in a water bath, fibril formation was monitored by the absorbance at 520 nm.

Incorporation of Alkali-Treated Collagen into Collagen Fibrils—Reconstituted fibrils (1 ml) were precipitated by centrifugation (Microcentrifuge MRX-150, Tomy, Tokyo) at the same temperature as the fibril-forming condition. The supernatants were removed and subjected to SDS-PAGE. The pellets were then rinsed in 1 ml of PBS at the same temperature and centrifuged again. One milliliter of a solution of sample buffer was added to the pellets. After heating at 100°C for 3 min, the samples were analyzed by SDS-PAGE. Because the mobility of the α chain of alkali-treated collagen in SDS-PAGE gel is slower than that of acid-soluble collagen (*17*), the amount of alkali-treated collagen and acid-soluble collagen could be assayed separately.

Detection of Conformational Changes by CD Spectroscopy and Proteolysis—A collagen solution (0.1 mg/ml) was placed in a spectropolarimeter Jasco-600 (Japan Spectroscopic), and the CD ellipticity at 221 nm was monitored at 37°C. In another method, trypsin was used to test for conformational changes in collagen (*19*). A set of collagen solutions (0.5 mg/ml) was incubated at 37°C. After a set period of incubation, trypsin was added to the solution and the mixtures were incubated at 20°C for 2 min. The enzymatic reaction was terminated by adding SDS, and SDS-PAGE was done. Native (in triple helical conformation) collagen is resistant to trypsin digestion.

Electron Microscopy—Collagen fibrils were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 30°C to avoid dissolving the alkali-treated collagen fibrils at low temperature, and then stained with 0.5% uranyl acetate (pH 4.4) and 2% phosphotungstic acid (pH 1.8). The samples were examined with an electron microscope as previously described (*20*).

Calculation of Interaction Score—The amino acid sequences of the bovine type I collagen α1 chain (*21*) and α2 chain (*22*) were used for calculations. Phe, Met, Val, Leu, Ile, and Tyr were examined for hydrophobic interactions, and Lys, Hyl (hydroxylysine), Arg, Glu, and Asp were examined for ionic interactions (*10, 18, 23*). To simplify the analysis, we assumed that the linear amino acid sequence could be analyzed as though the molecules were one-dimensional as described by Hulmes *et al.* (*23*) (Fig. 1A), although it is known that three collagen α chains are super coiled with a pitch of about 30 amino acid residues per turn (*10*).

The scoring system is as follows. In the case of hydrophobic interactions, amino acids with large hydrophobic side chains (Phe, Met, Val, Leu, Ile, and Tyr) were given a score of one. In the case of ionic interaction at pH 7.5, positively charged amino acids (Lys, Hyl, and Arg) were given a score of one and negatively charged amino acids (Glu and Asp) were given a score of minus one. Under acidic conditions (pH 4.5), positively charged amino acids (Lys, Hyl, Arg, and His) were given a score of one and negatively

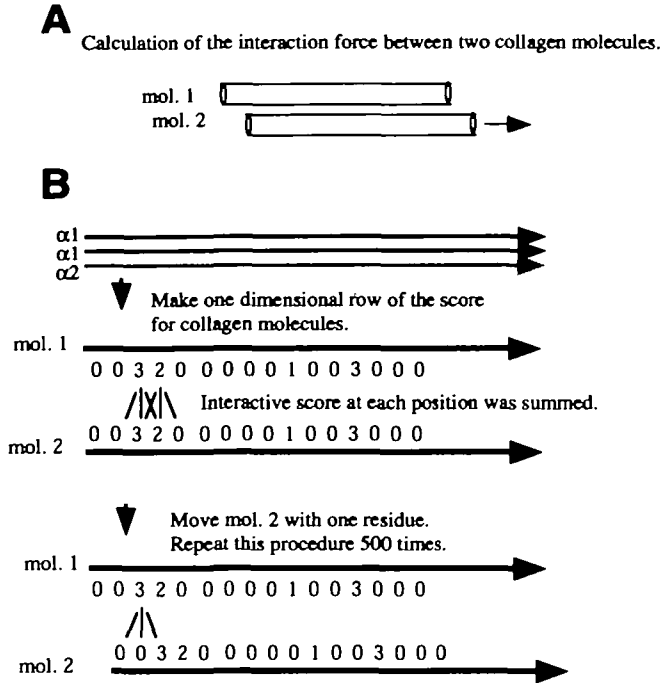

Fig. 1. **Protocol for calculating the interactive score between collagen molecules.** A, concept of the calculation; B, procedure for calculation.

charged amino acids Asp ($pK_a = 3.86$) and Glu ($pK_a = 4.25$) were given scores of -0.81 and -0.64, respectively. All other pairs were scored as zero. At each position of the amino acid sequence, the scores of three α chains (two $\alpha 1$ chains and one $\alpha 2$ chain) were summed and a tentative model collagen molecule with a row of 1,014 scores was formed (Fig. 1B).

We assume a range of ± 1 amino acid residue for each position involved in the interaction. In the case of hydrophobic interaction; at first, the score of the no. 1 molecule at the n position was multiplied by the score at the n position of the no. 2 molecule and the half-value of the score, $n-1$ and $n+1$ position, of the no. 2 molecule was multiplied by the score at the n position of the no. 1 molecule (Fig. 1B). The sum of three multiplications was the score of the hydrophobic interaction at the n position. The sum of the score at every interacting position was defined as the interaction score at the first position of the collagen molecule (0 stagger). Next, two sequences were moved past one another, one residue at a time, and at each position, the hydrophobic score was obtained by summing the scores for each amino acid pair in two collagen molecules. The interaction scores at positions 0 to 500 were calculated using Excel ver. 6 software (Microsoft, Seattle). Ionic interaction was also calculated by a similar method as described above. A negative score was taken as an attractive force and a positive score as a repulsive force.

RESULTS I

Self-assembling activity under physiological conditions (37°C, neutral pH in saline) is one of most prominent characteristics of fibrillar collagen. We reported earlier that collagen treated under alkaline conditions loses its ability to form fibrils *in vitro*, while the triple-helical conformation is retained (*16*). Digestion with MMP1, 8, or 13 also deprives collagen of its self-assembling ability while the collagen-specific triple helical conformation is retained (*24-26*). Alkaline treatment causes the shift of isoionic point of collagen (pI changes from 9.3 to 4.8) because of the deamination of Asn and Gln residues (*16*). By the MMP1, 8 or 13 digestion, collagen molecules are cut into 1/4 and 3/4 sections without amino acid modifications. We regarded those two collagen preparations as good probes to investigate the mechanism of inter-molecular collagen interactions. The participation of the ionic interaction can be estimated using alkali-treated collagen in which the Asn and Gln residues are changed to charged amino acids (Asp and Glu) without changing the conformation or size of the molecule. On the other hand, the effect of the size of the collagen molecules can be analyzed using MMP1-digested collagen fragments.

Effect of Alkali-Treated and MMP1-Digested Collagen Fragments on the Fibril Formation of Acid-Soluble Collagen at 37°C—First, we examined the effect of alkali-treated collagen or MMP1-digested collagen fragments on the fibril formation of acid-soluble collagen at neutral pH to observe the molecular interactions. We analyzed collagen interactions by adding alkali-treated collagen and MMP1-digested fragments into an acid-soluble collagen solution. When acid-soluble collagen (0.5 mg/ml) was incubated at 37°C (pH 7.4), fibril formation was completed within 20 min (Fig. 2, A and B, solid line). Acid-soluble collagen fibril formation was typical two-phase process, so-called nucleation phase followed by the growth phase (*12, 27, 28*). When an equal amount of alkali-treated collagen was added to the acid-soluble collagen solution, fibril formation at 37°C was drastically delayed (Fig. 2A), but the final turbidity measured after 2 h of incubation was the same as that of acid-soluble collagen in the absence of alkali-treated collagen ($A_{330} = 0.34$). Nucleation time (expressed as T_0) was prolonged from 9.5 to 19.5 min by adding an equal amount of triple-helical alkali-treated collagen. However, the rate of fibril formation (growth phase) was nearly the same for acid-soluble collagen alone (67 μg/min) and acid-soluble collagen in the presence of alkali-treated collagen (60 μg/min). On the other hand, MMP1-digested fragments, BSA, heat-denatured (h-d) acid-soluble, and h-d alkali-treated collagen (100°C for 2 min) had little effect on the fibril formation of native acid-soluble collagen (Fig. 2, A and B). When different amounts of alkali-treated collagen (final concentrations 0.1-1.0 mg/ml) were added to acid-soluble collagen at 37°C, T_0 was delayed in a manner dependent on the amount of added alkali-treated collagen (Fig. 2C). When the amount of alkali-treated collagen added (1 mg/ml) was twice that of the acid-soluble collagen (0.5 mg/ml), T_0 was delayed 17 min (from 11 min to 28 min), but G_{max} did not change (65 ± 5 μg/min).

Denaturation of Alkali-Treated Collagen in the Presence of Acid-Soluble Collagen at 37°C—An inhibitory effect of alkali-treated collagen on the fibril formation of acid-soluble collagen was observed when triple-helical alkali-treated collagen was added. This result indicates that only triple-helical alkali-treated collagen can interact with acid-soluble collagen. However, it has been reported that the denaturation temperature of alkali-treated collagen is lower than 35°C (*18*). Then, how can alkali-treated collagen affect the

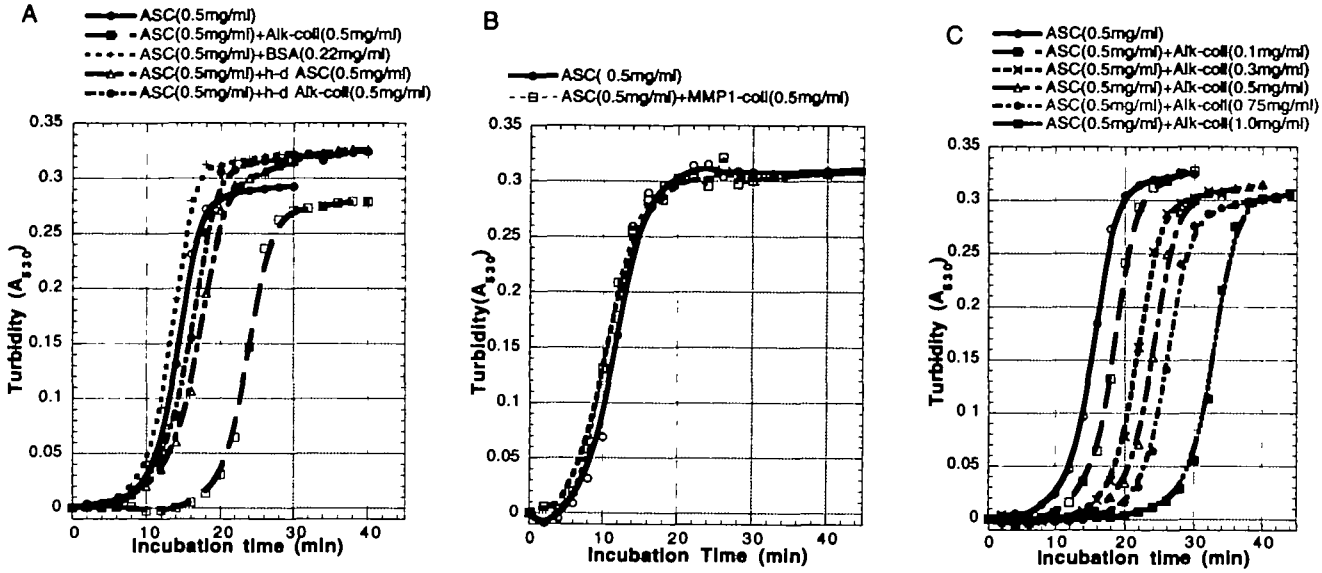

Fig. 2. **Effect of alkali-treated collagen and MMP1-digested fragments on the fibril formation of acid-soluble collagen at 37°C.** A: Fibril formation of acid-soluble collagen in the presence of native, denatured collagen and BSA was followed by measuring the turbidity at 530 nm. Added proteins are shown in the figure. ASC, Alk-coll, and MMP1-coll are acid-soluble collagen, alkali-treated collagen, and MMP1-digested collagen fragments, respectively. h-d: heat denatured. B: Fibril formation of acid-soluble collagen in the presence of MMP1-digested fragments was followed by measuring the turbidity at 530 nm. MMP1-coll is MMP1-digested collagen fragments. C: Fibril formation of acid-soluble collagen in the presence of different concentrations of alkali-treated collagens. Final concentrations of the added alkali-treated collagen are as indicated in the figure.

fibril formation of acid-soluble collagen at 37°C at which temperature the alkali-treated collagen is denatured? There are two possible explanations for the inhibitory effect of alkali-treated collagen at 37°C, one that the conformation of alkali-treated collagen is stabilized by co-incubation with acid-soluble collagen, and the other that alkali-treated collagen and acid-soluble collagen had already interacted with each other prior to warming.

To test whether or not the thermal stability of alkali-treated collagen is affected by the existence of acid-soluble collagen, conformational changes in alkali-treated collagen at 37°C were examined. Conformational changes in collagen were assayed by the trypsin treatment method (data not shown). The $\alpha 1$ chain of alkali-treated collagen was denatured within 2 min regardless of the presence of ASC. Acid-soluble collagen was stable at 37°C over the time examined (60 min). BSA that had no effect on fibril formation also had no effect on the thermal stability of the alkali-treated collagen. These results indicate that the heat stability of alkali-treated collagen is not affected by the presence of acid-soluble collagen. The CD analysis also indicated that the thermal stability of alkali-treated collagen was not affected by the presence of acid-soluble collagen.

Effect of Preincubation of Alkali-Treated Collagen and Acid-Soluble Collagen on Fibril Formation at 37°C— Because the presence of acid-soluble collagen did not change the thermal stability of alkali-treated collagen, next possibility was tested. We examined the effect of preincubation of alkali-treated collagen and acid-soluble collagen on fibril formation of acid-soluble collagen. Before the incubation at 37°C, a mixture of alkali-treated collagen (0.5 mg/ml) and acid-soluble collagen (0.5 mg/ml) was kept on ice for 5 min to 70 h, and fibril formation at 37°C was

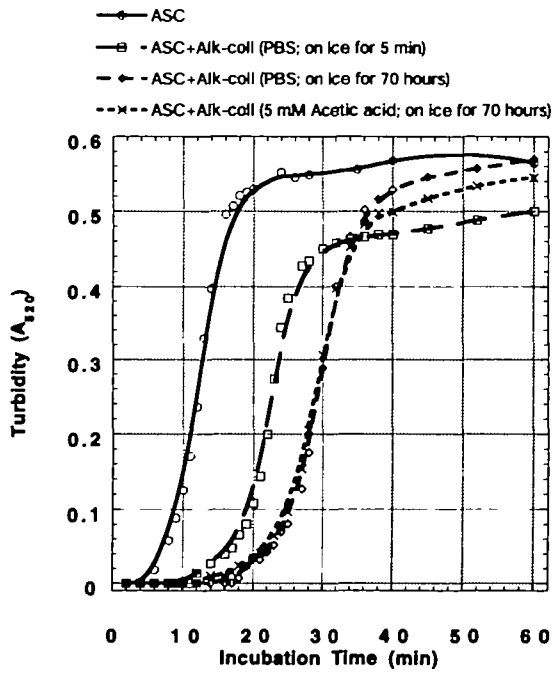

Fig. 3. **Effect of preincubation in the cold on fibril formation of acid soluble collagen.** Mixtures of acid-soluble collagen and alkali-treated collagen were kept on ice for the indicated period and the mixtures were then warmed to 37°C and the turbidity was measured.

monitored (Fig. 3). T_0 was prolonged depending on the period of preincubation on ice, while the rate of fibril formation did not change. Even when preincubation was performed in 5 mM acetic acid solution, a delay in T_0 was

observed as in PBS (Fig. 3). This suggests that the triple-helical form of alkali-treated collagen and acid-soluble collagen interact before warming up and that this interaction affects the features of fibril formation.

Effect of Alkali-Treated and MMP1-Digested Fragments on the Fibril Formation of Acid-Soluble Collagen at 30°C—We also examined fibril formation at 30°C. At 30°C, alkali-treated collagen and the larger MMP1-digested fragment (TCa) retain the triple-helical form (*16, 29*). When alkali-treated collagen (final concentration 0-1.0 mg/ml) was added to acid-soluble collagen (0.5 mg/ml), T_0 was delayed depending on the concentration of the added alkali-treated collagen (Fig. 4A). Unlike the case at 37°C, the growth rate was also inhibited by the addition of alkali-treated collagen. In the absence of alkali-treated collagen, the rate of fibril formation was 10.4 μg/min. When 0.1, 0.2, 0.3, 0.5, and 0.75 mg/ml of alkali-treated collagen was added, the G_{max} values were decreased to 7.9, 6.0, 4.9, 4.0, and 1.4 μg/min, respectively. When 1.0 mg/ml of alkali-treated collagen was added, G_{max} fell to less than 0.3 μg/min. However, the final turbidities (measured 6 days later) were almost the same under every condition (data not shown).

When MMP1-digested fragments (final concentration 0.5 mg/ml) were added to acid-soluble collagen (0.5 mg/ml), T_0 and the growth rate were slightly accelerated and the final turbidity was higher than when fibril formation was carried out in the absence of MMP1-digested collagen. When the incubation temperature was increased to 37°C after the completion of fibril formation at 30°C, the turbidity decreased to the same level as that of acid-soluble collagen in the absence of MMP1-digested fragments (Fig. 4B). This suggests that some parts of MMP1-digested fragments were incorporated into the fibril at 30°C.

Alkali-Treated Collagen Has Independent Effects on the Nucleation Phase and Growth Phase of Fibril Formation at 30°C—Unlike the inhibition effect at 37°C, at 30°C alkali-treated collagen affects not only the lag time (T_0) but also the growth rate. Therefore, we analyzed the change in T_0 and the growth rate separately. We examined the effect of adding alkali-treated collagen to acid-soluble collagen at a time when nucleation was almost completed. Alkali-treated collagen (0.8 mg/ml) was added to acid-soluble collagen after 20 min of incubation at 30°C (Fig. 5A), and the subsequent fibril formation was monitored (Fig. 5B). When acid-soluble collagen and alkali-treated collagen were co-incubated before warming (on ice), the growth rate of fibrils was 1.09 μg/min and T_0 was 400 min. When alkali-treated collagen was added after 20 min of incubation at 30°C, T_0 was shortened to 120 min but the G_{max} showed little change (1.16 μg/min). These results indicate that alkali-treated collagen in the triple-helical conformation interacts with acid-soluble collagen independently during the nucleation phase and the growth phase of fibril formation.

Incorporation of Alkali-Treated Collagen and MMP1-Digested Fragments into Collagen Fibrils at 30°C—Because the incubation experiments at 30°C suggested the interaction of alkali-treated collagen, MMP1-digested fragments and acid-soluble collagen, we examined the possibility that alkali-treated collagen and MMP1-digested fragments are incorporated into the fibrils of acid-soluble collagen. Alkali-treated collagen or MMP1-digested fragments were mixed with an equal amount of acid-soluble collagen on ice. Fibrils were allowed to form at 30 or 37°C

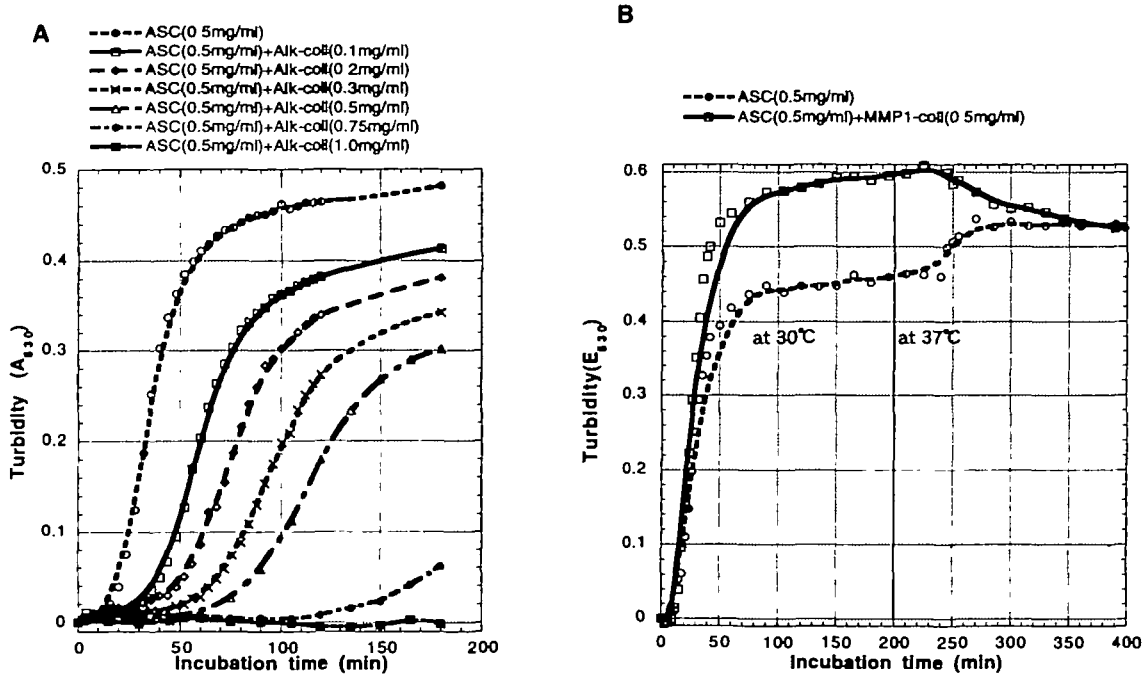

Fig. 4. **Effect of alkali-treated collagen and MMP1-digested fragments on the fibril formation of acid-soluble collagen at 30°C.** A: Fibril formation of acid-soluble collagen in the presence of different concentrations of alkali-treated collagen was followed by measuring the turbidity at 530 nm. Final concentrations of added alkali-treated collagen are shown in the figure. B: Fibril formation of acid-soluble collagen in the presence of MMP1-digested fragments was followed by measuring the turbidity at 530 nm. After incubation at 30°C for 200 min, the cuvette was warmed to 37°C.

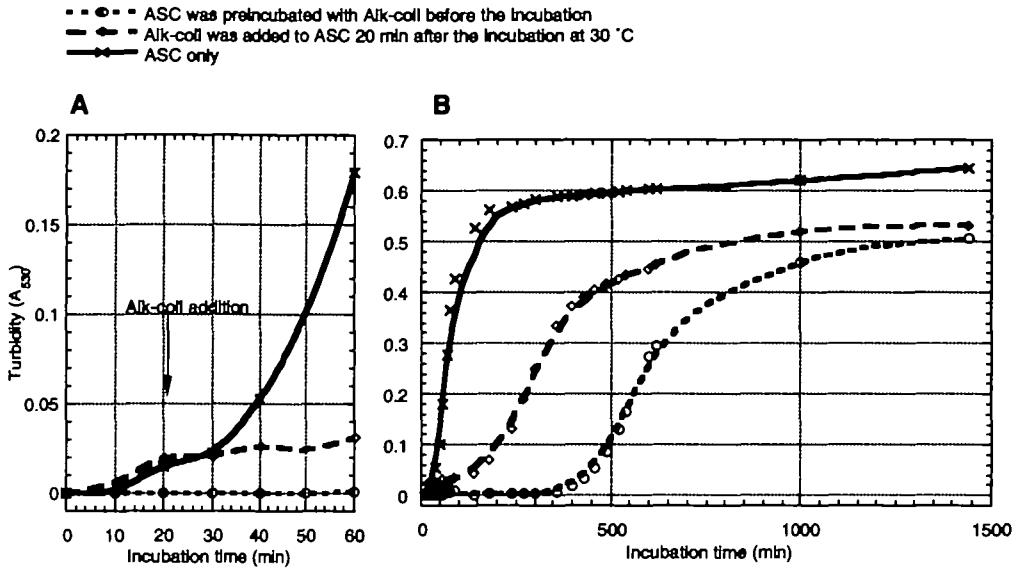

Fig. 5. **Effect of alkali-treated collagen added at the end of the nucleation phase.** Alkali-treated collagen (final concentration 0.8 mg/ml) was preincubated with acid-soluble collagen (0.5 mg/ml) on ice, or was added after 20 min of incubation at 30°C. In the case of acid-soluble collagen alone, the same volume of distilled water was added after 20 min of incubation. A, early stage of fibril formation is shown; B, the entire fibril formation process is shown.

for 24 h, after which the samples were centrifuged. The supernatant and precipitate (fibrils) were examined by SDS-PAGE, and the amounts of the $\alpha 1$ chains of each collagen were compared (Fig. 6). At 37°C, alkali-treated collagen and MMP1-digested fragments were not incorporated into the fibril fraction (data not shown). At 30°C, about 5% of the alkali-treated collagen and MMP1-digested fragments were incorporated into the fibril fraction (Fig. 6, lanes 2 and 4), while denatured alkali-treated collagen and BSA were not incorporated (Fig. 6, lanes 6 and 8). A 67-nm period banding pattern in the fibrils from acid-soluble collagen in the presence of alkali-treated collagen or MMP1-digested collagen formed at both 30 and 37°C could be observed by electron microscopy (data not shown).

RESULTS II

Fibril Formation of Alkali-Treated Collagen and MMP1-Digested Collagen Fragments—We have reported that the alkali-treated collagen loses its ability to form fibrils under neutral conditions, but that fibril formation is observed under acidic conditions in which ionic repulsive forces may be reduced (*16*). We examined the fibril-forming activity of alkali-treated collagen and MMP1-digested fragments under various conditions (Fig. 7). Because the alkali-treated collagen and MMP1-digested fragments are denatured at 37°C (*16, 29*), the incubation temperature was set at 30°C. At pH 4.5, fibril formation occurred at 30°C in the presence of 0.15 or 0.3 M NaCl and was complete within 2 min. Fibrils made of alkali-treated collagen were more fragile than fibrils of acid-soluble collagen formed at neutral pH. Fibril formation by MMP1-treated fragments was also observed at pH 4.5, but only in the presence of 0.3 M NaCl. Fibril formation by acid-soluble collagen was observed at pH 4.5 in the presence of 0.15 and 0.3 M NaCl; however, the rate of fibril formation was different from that of alkali-treated collagen (Fig. 7, A and B). In the presence of 0.3 M NaCl, fibril formation was complete within 2 min for all three collagen preparations. Fibril formation under these conditions began as soon as the cuvette was warmed without a lag time. On the other hand, in the presence of 0.15 M NaCl, the rate of fibril formation of acid-soluble collagen slowed, and more than 2 h needed for completion as in the case of the fibril formation of acid-soluble collagen at pH 7.5 in the presence of 0.15 M NaCl at 30°C. Under neutral conditions, alkali-treated collagen and MMP1-treated collagen did not form fibrils at any concentration of NaCl below 0.3 M. When the fibrils were cooled on ice, all the fibrils formed under acidic conditions melted (Fig. 7, A and B), while the acid-soluble collagen fibrils formed under neutral conditions were stable in cold condition (Fig. 7B). The stability of the fibrils in the cold depended on the pH of the collagen solution.

Fibril morphology was also examined under the electron microscope (Fig. 8). Fibrils formed from alkali-treated collagen at pH 4.5 in the presence of 0.15 or 0.3 M NaCl had a twisted fibrous structure 40–60 nm in diameter (Fig. 8, A, B, C, and D). Two or three fibrils were coiled together to form a super coiled fibril, so that the diameter of the fibril appeared larger than 60 nm. No collagen-specific D periodic banding patterns were found. Fibrils formed from acid-soluble collagen at pH 4.5 in the presence of 0.3 M NaCl (Fig. 8, G and H) were similar to the fibrils formed from alkali-treated collagen (Fig. 8, A, B, C, and D). Fibrils of MMP1-digested collagen also show a twisted structure under a scanning electron microscope (data not shown). Fibrils of acid-soluble collagen formed at pH 4.5 with 0.15 M NaCl show a linear morphology with a banding pattern of 67 nm (Fig. 8, E and F) like that of fibrils formed under neutral conditions (Fig. 8, I and J). The appearance of the banding pattern correlates with the existence of the lag phase during fibril formation (Fig. 7).

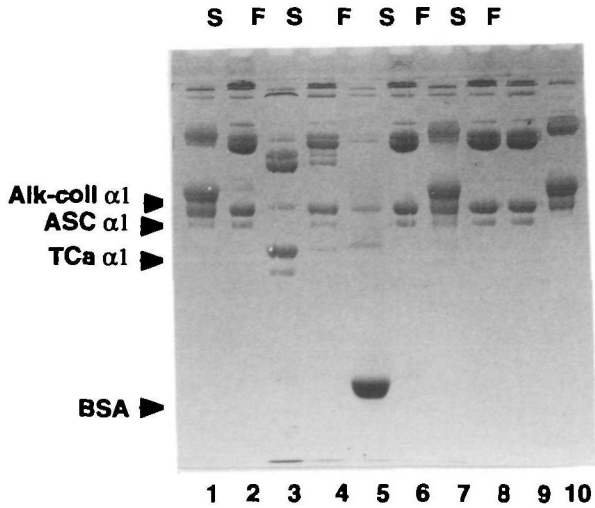

Fig. 6. **Incorporation of alkali-treated collagen and MMP1-digested fragment into fibrils.** Fibril formation was carried out at 30°C in an Eppendorf-type micro tube. After 20 h of incubation, the tube was centrifuged at 25-30°C at 14,000 rpm for 5 min. The supernatant and the precipitate dissolved in the same volume of supernatant were analyzed by SDS-PAGE (10% gel). Lanes 1 and 2, co-incubation of acid-soluble collagen and alkali-treated collagen; lanes 3 and 4, co-incubation of acid-soluble collagen and MMP1-digested fragments; lanes 5 and 6, co-incubation of acid-soluble collagen and BSA; lanes 7 and 8, co-incubation of acid-soluble collagen and heat-denatured alkali-treated collagen; lane 9, acid-soluble collagen without incubation; lane 10, alkali-treated collagen without incubation. Lanes 1, 3, 5, and 7 are the supernatant, and lanes 2, 4, 6, and 8 are the precipitate (fibrils). The positions of the α1 chain of collagen and BSA are shown. S indicates supernatant and F indicates fibril.

RESULTS III

Calculation of Interacting Forces between Collagen Molecules—The inhibitory effect of alkali-treated collagen on the fibril formation of acid-soluble collagen indicates that chemically modified collagen, which retains the triple helical conformation (log-like structure), and full length collagen can interact with acid-soluble collagen. This means that the linear alignment of the amino acid residues on the collagen molecule is important for the interaction between collagen molecules. Another characteristic feature of fibril formation is that the collagen forms fibrils only when the temperature of the solution is raised. This indicates that hydrophobic interactions that increase with increasing temperature are important for fibril formation, as pointed out before (*10*). Indeed, alkali-treated collagen that retains non-polar amino acids has more effect on the fibril formation of acid-soluble collagen than MMP1-digested fragments (Figs. 2 and 4). However, modification of polar amino acids by alkaline treatment disturbs fibril formation under neutral conditions (*16*). Both types of interaction are thought to be important for fibril formation; therefore, we calculated the interacting forces between collagen molecules using a method similar to that of Hulmes *et al.* (*23*). We used the recently determined amino acid sequence data of Shirai *et al.* (*22*) for α2 bovine type I collagen. The distribution of hydrophobic amino acid residues and charged amino acid residues in the model type I collagen molecules (linear molecule consisting of two α1 chains and one α2 chain) are shown in Fig. 9. In alkali-treated collagen,

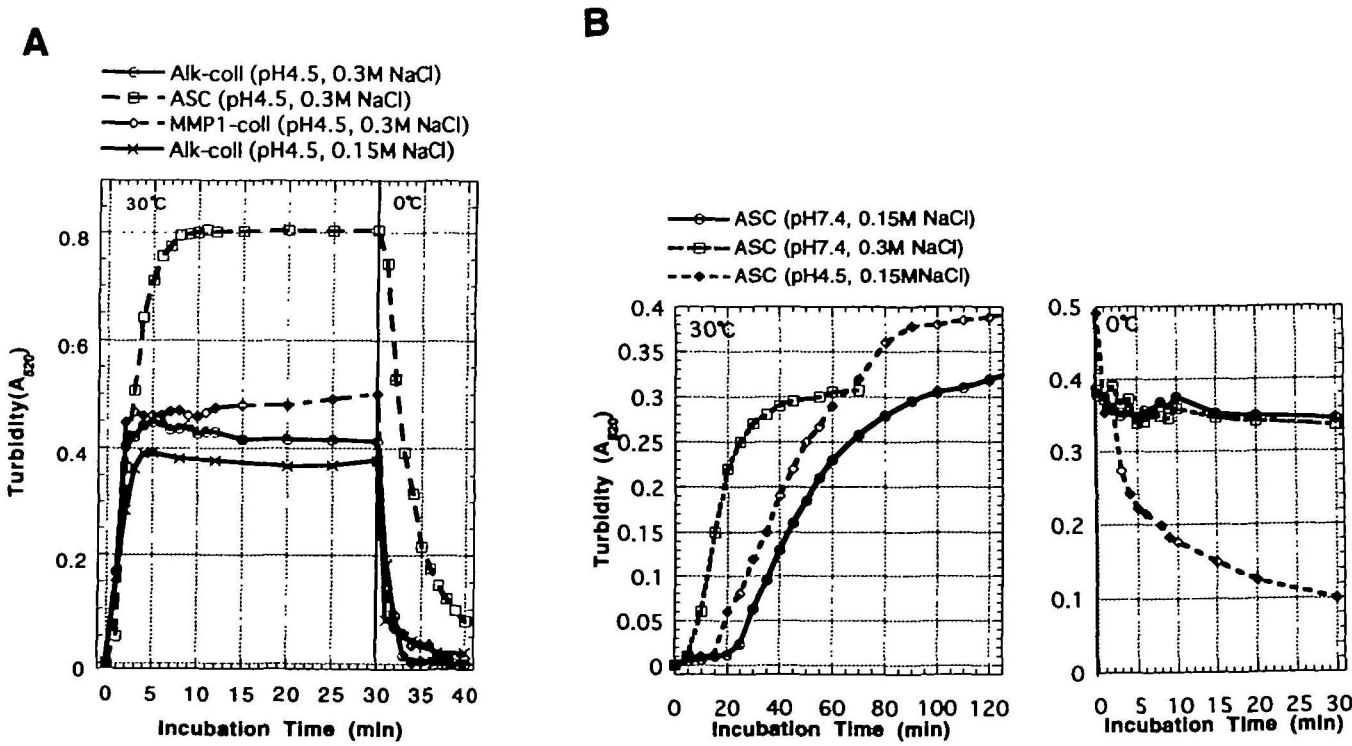

Fig. 7. **Fibril formation of collagen at different pH.** A: Acid-soluble collagen, alkali-treated collagen, and MMP1-digested fragments were incubated under the indicated conditions and the turbidity was measured. After 30 min, the cuvette was transferred onto ice and fibril melting was monitored. In this panel, the conditions under which fibrils were formed within 2 min are shown. B: Acid-soluble collagen was incubated under the indicated conditions. After 20 h of incubation, the cuvette was transferred onto ice.

all the Asn and Gln residues are converted to Asp and Glu by treatment with alkali (*16*). Under neutral conditions (pH 7.5), we assumed that His residues were not ionized. Under acidic conditions (pH 4.5), we assumed that 81% of the Asp and 64% of Glu residues were ionized.

Hydrophobic Interactions—Because the hydrophobic residues are not changed by alkali treatment, the interaction score of acid-soluble collagen and alkali-treated collagen was the same in our calculation. Though the positions of 3 to 500 residues are staggered in Fig. 10, several peaks in the interaction score appear. The D-staggered (position 235) peak is indicated in the figures by arrowheads. In addition to the D-staggered peak, peaks at 46, 85, and 148 (Fig. 10A, arrowheads) positions are prominent and those at 63, 103, 124, 171, and 190 positions are secondarily prominent. This profile is very similar to the calculation of Hulmes *et al.* (*23*) using only the α1 chain data and that of Hofmann *et al.* (*10*) which considered the triple-helical conformation of collagen. The peaks at positions 46, 85, 124, and 171 correspond to the 2/11D period that has been suggested before (*10, 23*).

When we examined the hydrophobic interaction score of TCa fragments (3/4 fragment of collagen containing the N-terminal side), a similar periodic pattern was observed including the D-staggered pattern (Fig. 10B). Experimentally, the TCa fragment interacted very weakly with acid-soluble collagen in fibril formation (Figs. 2B and 4B), and the activity of fibril formation was lost under neutral conditions. Lower value of the interaction score though 3 to 230 staggered positions (Fig. 10B) compared with alkali-treated collagen (Fig. 10A) may account for the weak interaction of the TCa fragments.

Ionic Interactions—Ionic interactions were calculated by multiplying the charged score of the amino acids both under neutral (Fig. 11A) and acidic conditions (Fig. 11B). In Fig. 11, the repulsive force was expressed as a positive value and the attractive force was expressed as a negative value. In every case, we could not find periodic patterns of

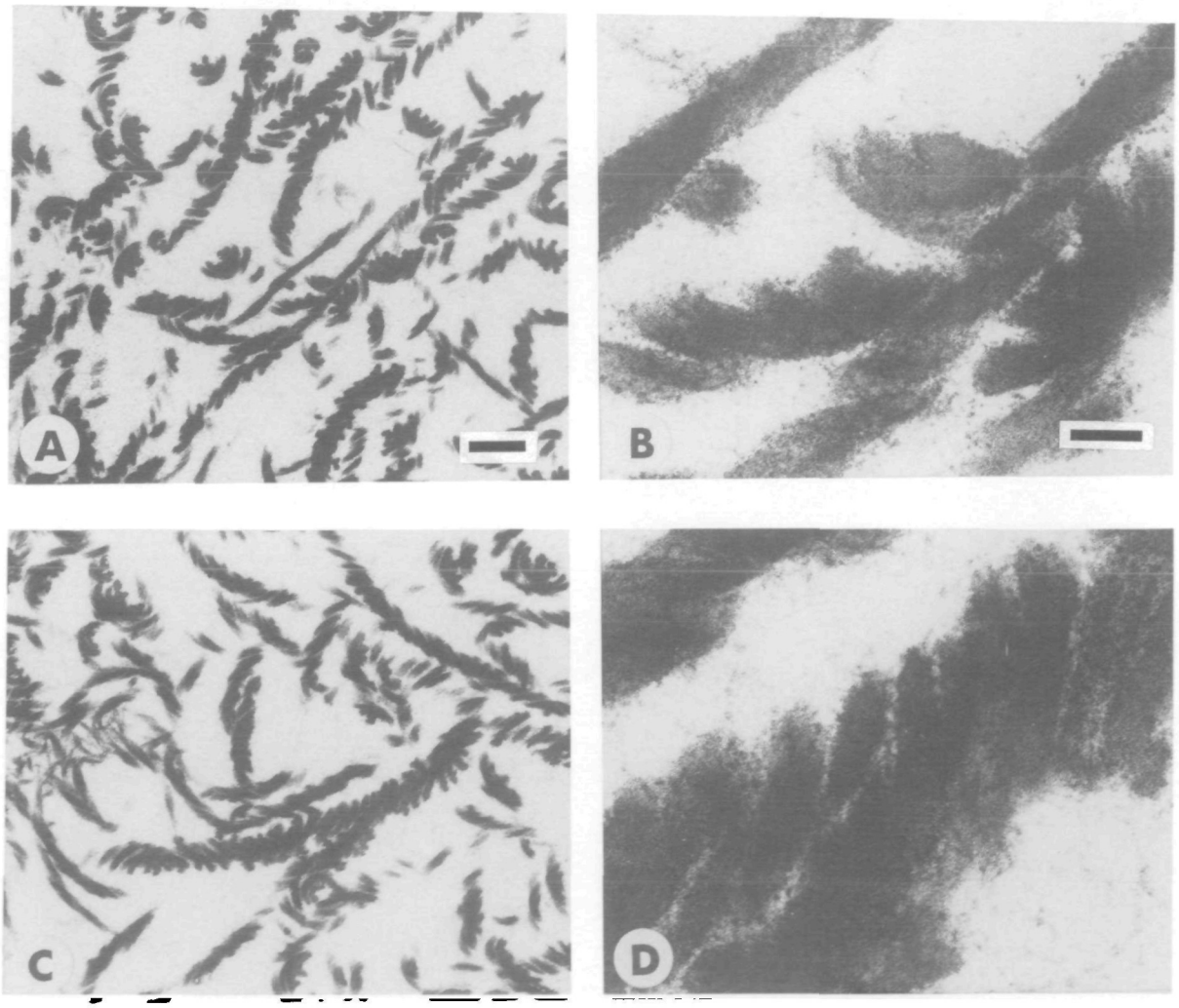

Fig. 8. **Electron microscopy of fibrils formed under various conditions.** Alkali-treated collagen or acid-soluble collagen in McIlvaine buffer was incubated at 30 C for 20 h. The fibrils were precipitated by centrifugation, fixed in warmed glutaraldehyde (30°C), and observed under an electron microscope. A and B, fibrils of alkali-treated collagen formed at pH 4.5 in the presence of 0.15 M NaCl; C and D, fibrils of alkali-treated collagen formed at pH 4.5 in the presence of 0.3 M NaCl; E and F, fibrils of acid-soluble collagen formed at pH 4.5 in the presence of 0.15 M NaCl; G and H, fibrils of acid-soluble collagen formed at pH 4.5 in the presence of 0.3 M NaCl; I and J, fibrils of acid-soluble collagen formed at pH 7.4 in the presence of 0.15 M NaCl. A, C, E, G, and I are same magnification; B, D, F, H, and J are same magnification. The bar in A indicates 500 nm and bar in B indicates 100 nm. (Continued on next page.)

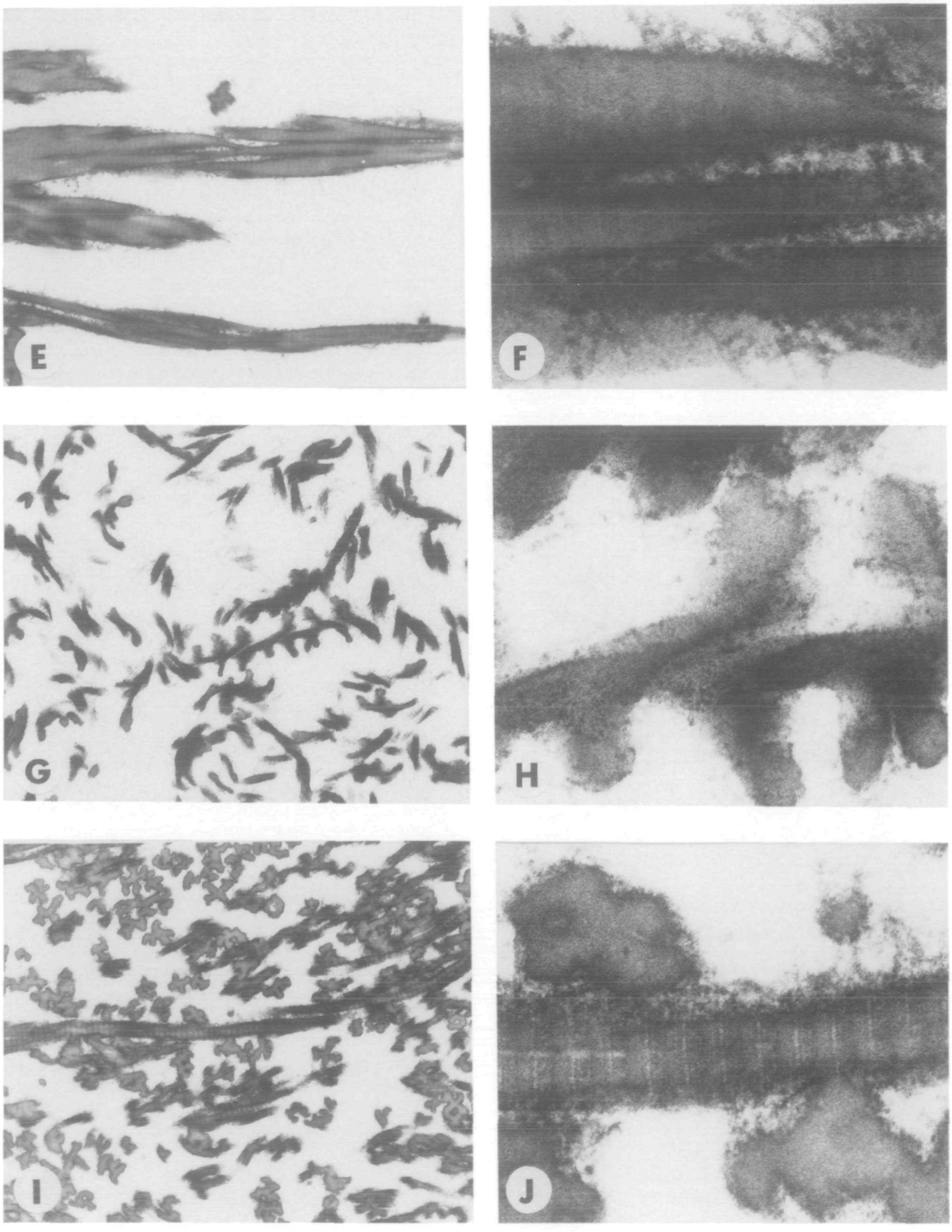

Fig. 8, E-J

interaction score through 4 to 500 staggered positions. Veis and George also did not find specific patterns for the distribution of charged residues in the amino acid sequences of collagen (*8*). Under neutral conditions, we compared the sum of the interaction scores for positions 4 to 500 staggered; the scores between acid-soluble collagens, between alkali-treated collagens, and between acid-soluble and alkali-treated collagen were +37, +5394, and −2220, respectively. The scores of 1 to 3 staggered positions were omitted because the scores were extremely

positive with same charged residues standing side by side. In the case of the interaction between acid-soluble collagens, the electrostatic force did not work attractively or repulsively. On the other hand, the electrostatic force between alkali-treated collagens was repulsive. This may explain the loss of fibril formation activity of alkali-treated collagen under neutral conditions. The attractive interaction between acid-soluble collagen and alkali-treated collagen was greatest in these comparisons. This may be related to the strong inhibitory effect of fibril formation of alkali-treated collagen under neutral conditions.

Under acidic conditions, the sums of the interaction scores for positions 4 through 500 staggered are as follows; between acid-soluble collagens, +7940, between alkali-treated collagens −586; and between acid-soluble and alkali-treated collagen, +1058. Attractive forces between alkali-treated collagen may account for its rapid fibril formation under acidic conditions (Fig. 7). In the case of acid-soluble collagen, we also observed fibril formation under acidic conditions, despite the repulsive force becoming dominant (Figs. 7, 8, and 11). The Asp and Glu residues lost their charges under acidic conditions and the hydrophobic interactions might be enhanced. The hydrophobic features of Asp and Glu, measured by their retention coefficients on reverse-phase HPLC, indicated them to be the most sensitive amino acids to the change in pH (*30*). We assumed that the hydrophobic interaction overcomes the electrostatic repulsive force under acidic conditions. Among the collagen preparations, only alkali-treated collagen showed an attractive interaction. Thus only alkali-treated collagen could form fibrils at lower NaCl concentrations under acidic conditions in which hydrophobic interactions are weak (Fig. 7A).

Carbohydrate—In collagen molecules, the presence of glucosylgalactosylhydroxylysine and galactosylhydroxylysine are known. These covalently linked carbohydrates are thought to affect the association of collagen molecules. In the $\alpha 1$ chain of type I calf skin collagen, carbohydrates are present at positions 103 and 697. In the $\alpha 2$ chain, carbohydrates are located at positions 103, 190, and 235 (*31*). There are microheterogeneities of the posttranslational modifications. For example, the extent of glycosylation at position 103 is though to be 0.4 mole (*31*). When we moved the model collagen molecules past one another, positions 45 (between position 235 and 190), 87 (190 and 103), 132 (235 and 103), 462 (697 and 235), 507 (697 and 190), and 594 (697 and 103) staggered have carbohydrates standing side by side. Positions other than 507 and 594 staggered are indicated in Fig. 10A by small arrows.

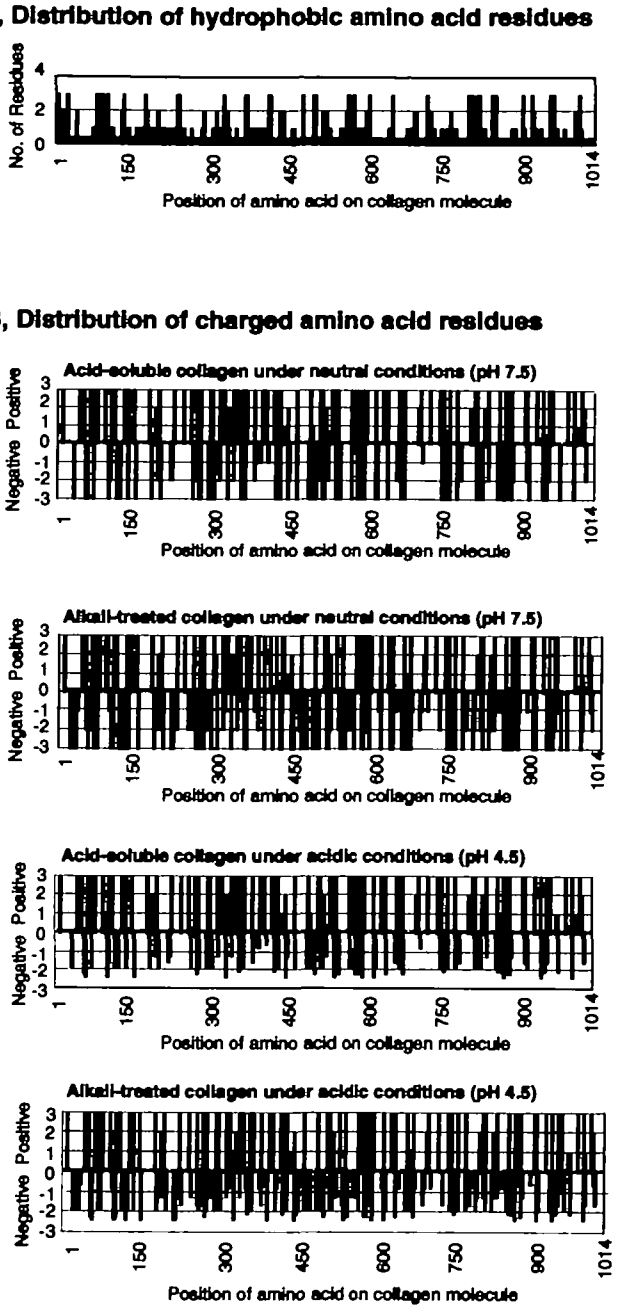

Fig. 9. **Axial distribution of amino acid residues along model collagen molecules consisting of two $\alpha 1$ chains and one $\alpha 2$ chain.** A, the distribution of hydrophobic residues; B, the distribution of charged residues of acid-soluble collagen and alkali-treated collagen at different pH.

DISCUSSION

We evaluated the interactive force of collagen molecules from the aspect of fibril formation using different kinds of collagen preparations. First, we examined the effect of alkali-treated collagen and MMP1-digested fragments on the fibril formation of acid-soluble collagen at neutral pH. Second, we tested the various conditions under which those collagen preparations would form fibrils and analyzed the morphology of the fibrils. Third, we calculated the interaction score using a model collagen molecule and compared the model with the experimental results.

Alkali-treated collagen alone does not form fibrils at neutral pH (*15, 16*), but has a drastic effect on the fibril formation of acid-soluble collagen. At 37°C, by adding alkali-treated collagen, the nucleation time (lag phase) was prolonged without an effect on the growth rate of fibril formation (Fig. 2). Based on calculations of the interaction score, the interaction between alkali-treated collagen and acid-soluble collagen can be enhanced electrostatically (Fig. 11). This interpretation is supported by the results of preincubation experiments, which indicated that the interaction of acid-soluble collagen and alkali-treated collagen had

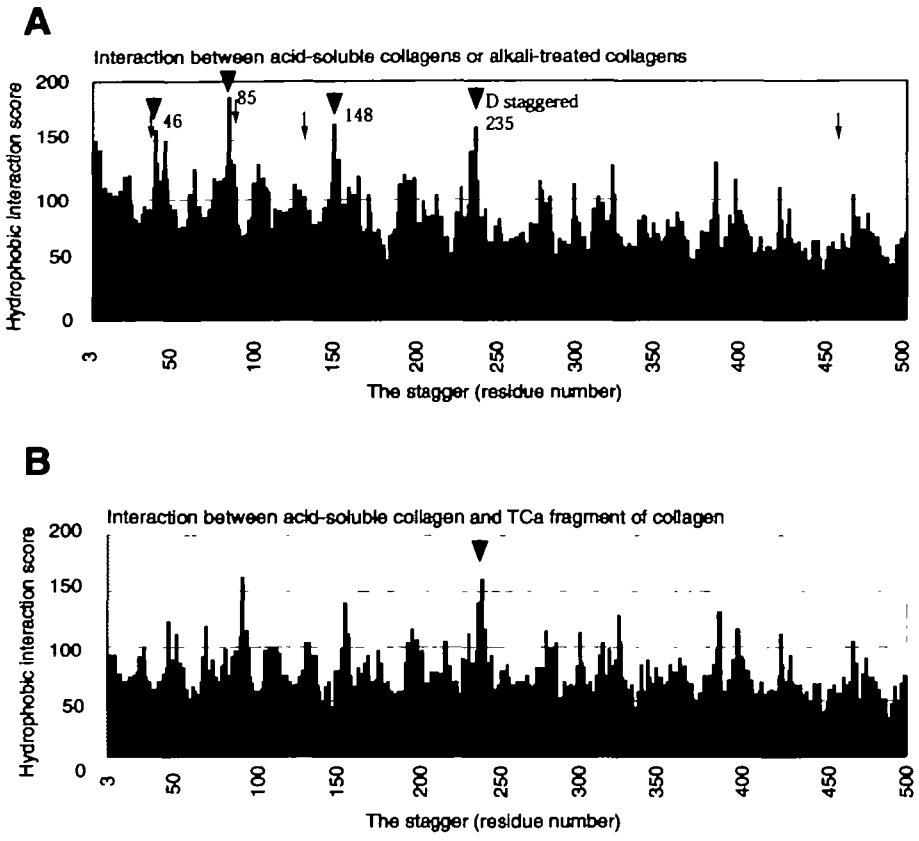

Fig. 10. **Variation in the hydrophobic interaction scores with the axial stagger of collagen molecules.** A: Interaction scores between acid-soluble collagens, alkali-treated collagens, or between acid-soluble collagen and alkali-treated collagen. Arrowheads and numbers show the position of prominent peaks of hydrophobic interaction. Small arrows show positions where the glycosylated hydroxylysine residues stand side by side. B: Interaction score between MMP1-digested collagen fragments (TCa) and acid-soluble collagen.

already occurred at the time of pre-incubation on ice (Fig. 3) at which electrostatic interactions are dominant over hydrophobic interactions. The complex of acid-soluble collagen and alkali-treated collagen does not act as a nucleus for fibril formation but rather disturbs the organized arrangement of acid-soluble collagens. By warming to 37°C, alkali-treated collagen is denatured and may lose its affinity for native collagen molecules. The prolonged T_0 caused by the addition of alkali-treated collagen at 37°C may reflect the time for the dissociation of the denatured alkali-treated collagen from acid-soluble collagen. The fact that denatured collagen has no effect on fibril formation (Fig. 2A) indicates that the collagen-specific triple helical conformation is essential for the interaction of collagen molecules. The triple helical conformation pushes the bulky amino acid outward and prepares the specific interacting surfaces for collagen molecules. A similar inhibitory effect of pepsin-treated collagen was reported by Hayashi and Nagai (*13*). They assumed that pepsin-treated collagen is a competitor of acid-soluble collagen.

Unlike the case of alkali-treated collagen, MMP1-digested fragments that also retain the collagen-specific triple helical conformation show no inhibitory effect on the fibril formation of acid-soluble collagen at 37°C (Fig. 2B). Because the distribution of charged amino acid residues is the same between acid-soluble collagen and MMP1-digested fragments, the only difference is the hydrophobic interaction, which is lowered by the shortening of molecular size. But the peak of hydrophobic interaction at the D-staggered position appears even in the MMP1-digested fragments in the calculation (Fig. 10B). This may be because partial parts of MMP1-digested fragment can associate in the fibril of acid-soluble collagen at 30°C without disturbing the assembly of the acid-soluble collagens, in spite of decreasing the hydrophobic interaction (Figs. 5B and 6). The accelerating effect of MMP1-digested fragments on fibril formation (Fig. 5B) may be understood as increasing the collagen concentration available for fibril formation.

At 30°C, not only T_0 but also the growth rate of fibrils is affected by the addition of alkali-treated collagen. The dual effect of alkali-treated collagen is independent (Fig. 5). When alkali-treated collagen is added at the end of the nucleation phase (20 min of incubation), T_0 is shortened to the same level as that without inhibitor (alkali-treated collagen), but the growth rate remained at the level with the inhibitor. Alkali-treated collagen retaining the triple-helical conformation serves as a competitor both in the phase of nucleus formation and the growth stage.

Under neutral conditions, the fibril formation of alkali-treated collagen does not proceed (*15, 16*). We considered that this failure to form fibrils might be due to the ionic repulsion of alkali-treated collagens whose isoelectric point is 4.8. To minimize the ionic repulsion, the fibril formation of alkali-treated collagen was examined at acidic pH (Fig. 7). Under acidic conditions at appropriate ionic strength, alkali-treated collagen forms a fibril-like precipitate at 30°C. Electron microscopic observation shows this precipitate to consist of fibers with a uniform diameter but without the collagen-specific D periodic banding pattern (Fig. 8, B and D). Surprisingly, the MMP1-digested collagen fragments and acid-soluble collagen, whose isoelectric point is basic (pI = 9.3), also formed fibrils at acidic pH (Fig. 7).

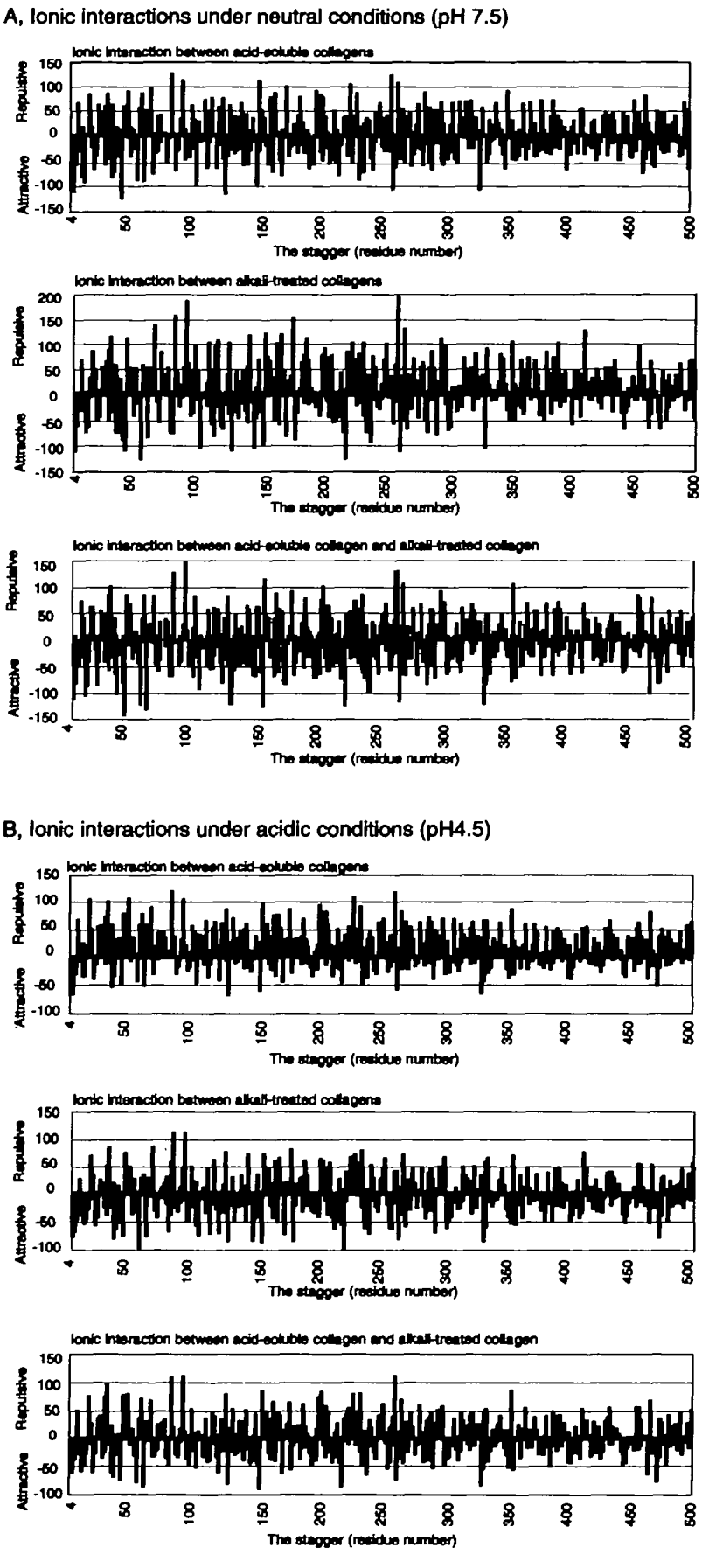

Fig. 11. **Variation in the ionic interaction scores with the axial stagger of collagen molecules at different pH.** A: Interaction scores between acid-soluble collagens, alkali-treated collagens, or between acid-soluble collagen and alkali-treated collagen under neutral conditions. B: Interaction scores between acid-soluble collagens, alkali-treated collagens, or between acid-soluble collagen and alkali-treated collagen under acidic conditions.

Schmitt *et al.* (*32*) have also reported the fibril formation of acid-soluble collagen at acidic pH. Under acidic conditions, the ionic interaction scores of acid-soluble collagens are strongly repulsive compared to those under neutral conditions (Fig. 11B). This may indicate that the increase in hydrophobic interactions overcomes the ionic repulsion under acidic conditions. In the presence of 0.3 M NaCl, all three collagen preparations form fibrils and the fibril formation is completed very fast (within 2 min) (Fig. 7). This can be explained as the decreased repulsive ionic strength by adding salt which masks the charged group of the amino acid by forming a diffuse double layer of counter ions (*10*). At lower ionic strength (0.15 M NaCl), at which electrostatic interactions increase, alkali-treated collagen forms fibrils as fast as in the case of 0.3 M NaCl. On the other hand, acid-soluble collagen requires an hour to form fibrils, and MMP1-digested fragments do not form visible fibrils (Fig. 7). This result is in agreement with the calculated ionic interaction in which only alkali-treated collagen shows an attractive interaction under acidic conditions. Fibrils of acid-soluble collagen formed in neutral solution are stable when cold (Fig. 7B), while all fibrils formed under acidic conditions melt when cooled (Fig. 7A). This may be due to an inhibition in the formation of crosslinks between collagen molecules under acidic conditions.

There were two prominent morphological differences between fibrils of alkali-treated collagen and a typical fibril of acid-soluble collagen formed at neutral pH. The fibrils of alkali-treated collagen lack the D periodic banding pattern and are twisted together into a coiled morphology (Fig. 8, A and C). The shape of the acid-soluble collagen fibrils formed under acidic conditions changes depending on the ionic strength of the solution. At lower ionic strength (0.15 M NaCl), the fibrils show the banding pattern and a non-coiled morphology (Fig. 8, E and F). At higher ionic strength (0.3 M NaCl), the fibrils show no banding and are coiled (Fig. 8, G and H). The fibrils of the non-banded type were formed within 2 min after warming, while the time course of fibril formation of the D-periodic banding type showed a lag phase (Fig. 7). The calculated electrostatic interaction score under conditions that produce the banding type of fibril is lower than that that produces the non-banding type (Fig. 11) in the same collagen preparation. These facts suggest that an interaction between molecules that is too strong can result in a failure to form the D periodic banding collagen fibrils. The calculated interactive score supports the forming of D-staggered fibrils driven by hydrophobic interactions, but in addition to the 1-D stagger position, there are some attractive peaks of hydrophobic interaction (Fig. 10). Under highly interactive conditions, collagen molecules may assemble randomly between the nearest interactive positions. On the other hand, under relatively weak attractive conditions, the molecule may have a chance to grope for a suitable position. We do not yet fully understand why collagen molecules choose the 1D-staggered peak out of several peaks of hydrophobic interaction, but there are two possible explanations. One is the existence of carbohydrate. We assume that carbohydrates will disturb the assembly of collagen molecules where the glycosylated residues stand side by side. In "RESULTS III", we found this situation to exist at positions of 45, 87, 132, 462, 507, and 594 staggered, and that the 45 and 87 staggered positioning is very close to the attractive peak of hydrophobic interaction. This may be help the D-period to be selected as the first choice during the association of collagen molecules. The other possibility is that the triple helical pitch, which we did not consider in our calculations, is responsible for the D-staggered assembly. Hoffman *et al.* suggested that the ionic interaction shows a prominent signal when set at 36° for the triple helical angle (30 amino acid residues per turn) (*10*). The alkali-treated collagen under acidic conditions (also MMP1-digested collagen and acid-soluble collagen under acidic conditions in the presence of 0.3 M NaCl) may have a different triple helical angle from 36° and its fibril may not be stable in a linear structure, but may form a twisted (super coiled) structure.

Our results indicate that very delicate control is needed to form collagen fibrils with a D period, and we observed that there is lag phase when the D periodic-linear and non-coiled fibrils are formed. This may be an important feature *in vivo* where collagen fibrils sometimes must be formed in places remote from the collagen-producing cells. The lag phase must be essential to allow the collagen molecules to be conveyed in a soluble form to a suitable site in the tissue. It has been suggested that procollagen C-proteinase controls collagen fibril formation by cutting the C-propeptide of collagen (*5-7*). The lag phase mechanism would be also utilized *in vivo* to control fibril formation.

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REFERENCES

1. Adachi, E., Hopkinson, I., and Hayashi, T. (1997) Basement-membrane stromal relationships: Interactions between collagen fibrils and the lamina densa. *Int. Rev. Cytol.* **173**, 73-156
2. Cleary, E.G. (1996) Skin in *Extracellular Matrix Vol. 1. Tissue Function* (Comper, W.D., ed.) pp. 77-109, Harwood Academic Publishers, Australia
3. Viidic, A. (1996) Tendons and ligaments in *Extracellular Matrix Vol. 1. Tissue Function* (Comper, W.D., ed.) pp. 303-327, Harwood Academic Publishers, Australia
4. Gross, J. and Kirk, D. (1958) The heat precipitation of collagen from neutral salt solutions: some rate-regulating factors. *J. Biol. Chem.* **233**, 355-360
5. Prockop, D.J. and Hulmes, D.J. (1994) Assembly of collagen fibrils *de novo* from soluble precursors: polymerization and copolymerization of procollagen, pN-collagen, and mutated collagens in *Extracellular Matrix Assembly and Structure* (Yurchenco, P.D., Birk, D.E., and Mecham, R.P., eds.) pp. 47-90, Academic Press, San Diego
6. Kadler, K.E., Hojima, Y., and Prockop, D.J. (1987) Assembly of collagen fibrils *de novo* cleavage of the type I pC-collagen with procollagen C-proteinase. *J. Biol. Chem.* **262**, 15696-15701
7. Kadler, K.E., Hojima, Y., and Prockop, D.J. (1990) Collagen fibrils *in vitro* grow from pointed tips in the C- to N-terminal direction. *Biochem. J.* **268**, 339-343
8. Veis, A. and George, A. (1994) Fundamentals of interstitial collagen self-assembly in *Extracellular Matrix Assembly and Structure* (Yurchenco, P.D., Birk, D.E., and Mecham, R.P., eds.) pp. 15-46, Academic Press, San Diego
9. Kobayashi, K., Niwa, J., Hoshino, T., and Nagatani, T. (1992) Electron microscopic visualization of collagen aggregates without chemical staining. *J. Electron Microsc. (Tokyo)* **41**, 235-241
10. Hofmann, H., Fietzek, P.P., and Kühn, K. (1978) The role of polar and hydrophobic interactions for the molecular packing of type I collagen: A three-dimensional evaluation of the amino acid sequence. *J. Mol. Biol.* **125**, 137-165

11. Hayashi, T. and Nagai, Y. (1972) Factors affecting the interactions of collagen molecules as observed by *in vitro* fibril formation. I. Effects of small molecules, especially saccharides. *J. Biochem.* **72**, 749-758
12. Hayashi, T. and Nagai, Y. (1973) Factors affecting the interactions of collagen molecules as observed by *in vitro* fibril formation. II. Effects of species and concentration of anions. *J. Biochem.* **74**, 253-262
13. Hayashi, T. and Nagai, Y. (1974) Factors affecting the interactions of collagen molecules as observed by *in vitro* fibril formation. III. Non-helical regions of the collagen molecules. *J. Biochem.* **76**, 177-186
14. Prockop, D.J. and Fertala, A. (1998) Inhibition of the self-assembly of collagen I into fibrils with synthetic peptides. *J. Biol. Chem.* **273**, 15598-15604
15. Rauterberg, J. and Kühn, K. (1968) The renaturation behavior of modified collagen molecules. *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 611-622
16. Hattori, S., Adachi, E., Ebihara, T., Shirai, T., Someki, I., and Irie, S. (1999) Alkali-treated collagen retained the triple helical conformation and the ligand activity for cell adhesion via $\alpha 2\beta 1$ integrin. *J. Biochem.* **125**, 676-684
17. Kuwaba, K. and Fujii, T. (1996) Identification of type III collagen-derived polymeric components in commercial lime-processed hide gelatins. *J. Photogr. Sci.* **44**, 62-65
18. Hattori, S., Sakai, K., Watanabe, K., and Fujii, T. (1996) The induction of metachromasia and circular dichroism of Coomassie brilliant blue R-250 with collagen and histone H1 is due to the low content of hydrophobic amino acid residues in these proteins. *J. Biochem.* **119**, 400-406
19. Bruckner, P. and Prockop, D.J. (1981) Proteolytic enzymes as probes for the triple-helical conformation of procollagen. *Anal. Biochem.* **110**, 360-368
20. Adachi, E. and Hayashi, T. (1986) *In vitro* formation of hybrid fibrils of type V collagen and type I collagen. Limited growth of type I collagen into thick fibrils by type V collagen. *Connect. Tissue Res.* **14**, 257-266
21. Bornstein, P. and Traub, W. (1979) The chemistry and biology of collagen in *The Proteins* Vol. IV (Naurath, H. and Hill, R.L., eds.) pp. 411-632, Academic Press, New York
22. Shirai, T., Hattori, S., Sakaguchi, M., Inouye, S., Kimura, A., Ebihara, T., Irie, S., Nagai, Y., and Hori, H. (1998) The complete cDNA coding sequence for the bovine proα2(I) chain of type I procollagen. *Matrix Biol.* **17**, 85-88
23. Hulmes, D.J.S., Miller, A., Parry, D.A.D., Piez, K.A., and Woodhead-Galloway, J. (1973) Analysis of the primary structure of collagen for the origins of molecular packing. *J. Mol. Biol.* **79**, 137-148
24. Gross, J. and Nagai, Y. (1965) Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. *Proc. Natl. Acad. Sci. USA* **54**, 1197-1204
25. Woodlley, D.E. (1984) Mammalian collagenases in *Extracellular Matrix Biochemistry* (Piez, K.A. and Reddi, A.H., eds.) pp. 119-157, Elsevier, New York
26. Nagase, H., Barret, A.J., and Waessner, J.F. (1992) Nomenclature and glossary of the matrix metalloproteinases. *Matrix Suppl.* **1**, 421-424
27. Comper, W.D. and Veis, A. (1977) The mechanism of nucleation for *in vitro* collagen fibril formation. *Biopolymers* **16**, 2113-2131
28. Gelman, R.A., Williams, B.R., and Piez, K.A. (1979) Collagen fibril formation. Evidence for a multistep process. *J. Biol. Chem.* **254**, 180-186
29. Sakai, T. and Gross, J. (1967) Some properties of the products of reaction of tadpole collagenase with collagen. *Biochemistry* **6**, 518-528
30. Meek, J.L. (1980) Prediction of peptide retention times in high-pressure liquid chromatography on the basis of amino acid composition. *Proc. Natl. Acad. Sci. USA* **77**, 1632-1636
31. Kivirikko, K.I. and Myllylä, R. (1979) Collagen glycosyltransferases in *International Review of Connective Tissue Research* Vol. 8 (Hall, D.A. and Jackson, D.S., eds.) pp. 23-72, Academic Press, New York
32. Schmitt, F.O., Gross, J., and Highberger, J.H. (1953) A new particle type in certain connective tissue extracts. *Proc. Natl. Acad. Sci. USA* **39**, 459-470