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Characteristics of Type IV Collagen Unfolding Under Various pH Conditions as a Model of Pathological Disorder in Tissue

Akio Shimizu[1,*], Kenichi Kawai[1], Miki Yanagino[1], Toshiko Wakiyama[1], Minoru Machida[2], Kohji Kameyama[3] and Zenya Naito[3]

[1]Department of Environmental Engineering for Symbiosis, Faculty of Engineering, Soka University; [2]Cancer Screening Technology Division Research Center For Prevention and Screening, National Cancer Center; and [3]Department of Pathology, Nippon Medical School, Tokyo, Japan

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The overall structure of type IV collagen is the same at neutral and acidic pH, as determined by circular dichroism spectra. The heating rate dependence of denaturation midpoint temperature (T_m) shows that type IV collagen is unstable at body temperature, similarly to type I collagen. The heating rate dependence of T_m at neutral pH has two phases, but that at acidic pH apparently has a single phase. The T_m of the first phase (lower T_m) at neutral pH is consistent with that at acidic pH, and the activation energy of these phases is consistent, within experimental error. The triple helix region of type IV collagen corresponding to the second phase (higher T_m) at neutral pH is thermally stable when compared to the triple helical structure at acidic pH. At acidic pH, as the loosely packed and unstable region has spread throughout the whole molecule, the thermal transition is thought to be cooperative and is observed as a single phase. Structural flexibility is related to protein function and assembly; therefore, the unstable structure and increased flexibility of type IV collagen induced at acidic pH may affect diseases accompanied by type IV collagen disorder.

Key words: collagen type IV, effect of solution pH, thermal unfolding.

Abbreviations: UV, ultraviolet; CD, circular dichroism; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Collagen, the most abundant animal protein, forms a triple helix (*1–5*). Various types of collagen have been reported, and they form the matrix of bone, skin, basement membrane and other tissues (*2*, *6*, *7*). Type IV collagen is an element of basement membrane (*8*, *9*), and is considered to be an important factor in various diseases, such as arteriosclerosis, the acidic environment of cancer and basement membrane illness (*10–12*). Therefore, understanding the structural stability of collagen is important in understanding the mechanism of these illnesses. The extracellular pH of microenvironment in pathological lesion (cancer, inflammation and atherosclerosis) in tissue is often lower than in normal tissue (*11*, *13–18*). In some cases, it is pointed out that the acidic conditions around pH5 or below that are important in relation to pathological tissue (*13–18*). Under such acidic environment in the wide range, the turnover and degradation of collagen IV as main component basement membrane are examined (*17*, *18*).

The thermal denaturation of collagen is very complex, and the thermal stability of collagen expressed in its denaturation midpoint temperature (T_m) depends significantly on environmental conditions, pH, presence of salts, denaturants, *etc*. (*12*, *19–21*). Therefore, it is important to clarify the differences in the thermodynamic properties of type IV collagen at acidic and neutral pH as models of normal and pathological tissues, respectively. However, our understanding of the thermodynamic parameters and mechanisms of the thermal denaturation of type IV collagen is poor when compared with type I collagen (the most abundant animal protein, forming the matrix of bone, skin and other tissues) (*19*, *22–25*).

It has previously been reported that the characteristic element of all collagens is the triple helix formed by three subunit chains, and that assembly is based on the repetition of typical Gly-X-Y sequences (*3–6*, *26*, *27*). In addition, the type IV collagen triple helical domain (repetition of typical Gly-X-Y sequence), which is ~1,400 residues long, contains more than 20 imperfections (*28–32*). In addition to affecting molecular and higher order structure, breaks in the $(Gly\text{-}X\text{-}Y)_n$ pattern can also serve as interaction sites of biological importance. For example, the sequence of the α1 chain of type IV collagen contains a break that was shown to promote the adhesion of melanoma, ovarian carcinoma and Jurkat cells (*33*, *34*). Structural and functional consequences may be influenced by the nature of the break and by the sequence of surrounding residues (*35*).

Despite these imperfections, the triple helix of type IV collagen is stable and the T_m is comparable to that of type I collagen (*20*, *36*). This is explained by the high content of hydroxyproline in type IV collagen. Furthermore, type I collagen is thermally unstable at body temperature (*19*), while exposure of either collagen

*To whom correspondence should be addressed. Tel: +81-426-91-2317, Fax: +81-426-91-2317, E-mail: shimizu@t.soka.ac.jp

or procollagen to ultraviolet (UV) radiation reduces T_m (*36*). The thermal stability of proteins has long been a practical concern, because this is one of most important factors limiting their usefulness. Protein conformation ensembles are undoubtedly affected by temperature, but it is uncertain whether temperature affects the folding and unfolding mechanisms by influencing conformation (*37*). Flory and Garrett (*38*) reported that the thermal denaturation of collagen is a phase transition involving the melting of crystalline regions. Their explanation was widely accepted, and the word melting is commonly used synonymously with denaturation or unfolding to denote the helix-coil transition of collagen. Many researchers have investigated this issue, having assumed the thermal denaturation of collagen to be an equilibrium state. On the other hand, the T_m value of collagen is known to be largely dependent on heating rate (*19*). More recently, Leikina *et al*. (*19*) reported that the equilibrium denaturation time of collagen is >10 days. From these reports and detailed experimental results, Miles proposed that the thermal denaturation of collagen analysed in the equilibrium process should be analysed in a kinetics process (*39*).

In the present article, we clarify the thermal denaturation process and detail the thermodynamic properties of type IV collagen at neutral and acidic pH (pH 7.2 and 4.3) as models of normal and pathological organization, respectively. In this experiment, we used circular dichroism (CD) spectroscopy, as CD provides information about secondary structure. Specifically, we investigated differences in the heat denaturation process at neutral and acidic pH by studying the heating rate dependence of T_m and the time course at fixed temperatures. Furthermore, from the time course at different heating rates, we calculated the activation energy.

MATERIALS AND METHODS

Type IV collagen purified from bovine placenta was purchased from Korken (Tokyo, Japan). This type IV collagen was supplied dissolved in HCl solution (pH 3) at a concentration of 0.3% wt/v. Collagen was treated with pepsin, and the 7S and NC1 domains were cleaved. Other extra-pure grade chemical reagents, NaCl, Na_2HPO_4, and NaH_2PO_4, and urea of biochemical grade were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

CD measurements were made using a Jasco J-805 spectropolarimeter (Jasco Engineering, Tokyo, Japan) with a thermoelectrically controlled cell holder. The light source used in the CD spectropolarimeter was a 150 W xenon lamp. CD samples were prepared in 50 mM sodium phosphate buffer at pH 7.2 or pH 4.3 (final collagen concentration was 0.03 or 0.006% wt/v). Quartz cells with a path length of 1 mm were used. The temperature of sample solution was controlled using an F25 refrigerated/heating circulator (Julabo Inc., Seelbach, Germany), which is able to regulate temperature with an accuracy of ±0.01°C. The temperature probe was inserted into the sample solution and the temperature of sample solution was measured and controlled directly. Heating/cooling rates of 1.0, 0.5, 0.01 and 0.05 C min^{-1} were maintained using the temperature control program supplied with the CD equipment. Thermal denaturation curves were obtained by measuring ellipticity at 222 nm, and T_m was analysed by first derivation.

Purity and fragmentation after UV irradiation was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7% SDS-PAGE). Chain composition was determined using a Waters C18 reverse-phase column. Peaks were separated by HPLC (Shimadzu LC-6AD) using an acetonitrile gradient (20–45%).

The rate of collagen unfolding at different temperatures was measured directly by CD with 0.03% collagen solution. The native collagens at pH 4 and 7 were heated rapidly to the set temperature, and CD value was recorded as a function of time. An empty cell and the solution not containing collagen were pre-incubated to a set temperature. Before measurement, collagen was added to the solution, and was then transferred to the cell. After collagen was added to the solution, the time to measurement start was ~15 s.

RESULTS

CD Spectrum of Type IV Collagen at Neutral and Acidic pH—Figure 1 shows the CD spectrum in the far-UV region of type IV collagen at neutral (pH 7.2) and acidic (pH 4.3) pH at 37°C. The two CD spectra overlapped completely. These spectra exhibit a maximum at 222 nm and a minimum at 198 nm, which are typical for a collagen triple helix (*40*, *41*). Rpn values (*42*, *43*), which are defined as the absolute values of the ratio between dichroic intensity of the positive maximum over that of the negative maximum, were 0.096 and 0.098 for neutral and acidic pH, respectively. This strongly indicates a triple helical structure spanning the entire

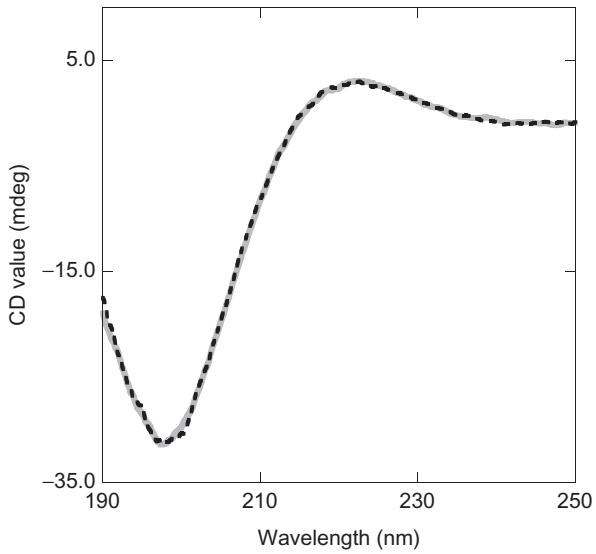

Fig. 1. **Circular dichroism spectra of 0.03% type IV collagen at 37°C in 50 mM phosphate buffer at neutral pH (pH 7.2, dotted line) and acidic pH (pH 4.3, solid line).**

molecule from the N to the C terminus at both neutral and acidic pH (*21*, *42*).

Effects of UV Irradiation on Type IV Collagen Structure—Previous papers have indicated that collagen is damaged by UV irradiation (*19*, *20*, *23*, *43*, *44*). For type I collagen, irradiation at 215 and 220 nm was equally effective, irradiation at 230 nm was less effective, and irradiation at 240–260 nm had no effect over 15 h at 4°C (*23*). In addition, UV irradiation damage can be reduced by increasing protein concentration (*23*). It has also been reported that the thermal transition temperature is decreased by UV irradiation, although the shape of the CD spectrum is not changed. Therefore, in this experiment, we carefully investigated the UV irradiation effects caused by the light source (222 nm) of the CD spectrometer. One sample was irradiated at 222 nm in the CD spectrometer for 30 min, while another sample was not irradiated before measurement. After starting the measurement, thermal denaturation was continuously monitored based on CD value at 222 nm in both samples (70 min, sample was heated from 25 to 60°C at a heating rate of 0.5°C min^{-1}). These two thermal transition curves were overlapped completely at each pH, and a shift in transition curve was not observed. Next, to minimize the effects of UV, CD data was acquired at several points in the thermal denaturation process, and was not continuously irradiated. In this case, samples were irradiated with UV for ~1 min at each measuring point, and the light was intercepted at all other times (sample was continuously heated at 0.5°C min^{-1}). Total UV irradiation time was 5–7 min. As shown in Fig. 2, these observed points completely overlapped the transition curve obtained with continuous UV irradiation (heating rate of 0.5°C min^{-1} for a total of 70 min).

SDS-PAGE was used to investigate whether the polypeptide chains were cleaved by UV irradiation. No differences in SDS-PAGE patterns before and after UV irradiation were observed (data not shown).

Refolding of Thermally Denatured Type IV Collagen—Thermal unfolding and refolding curves monitored at 222 nm at temperatures ranging from 5°C to 70°C, with a heating/cooling rate of 0.5°C min^{-1}, are shown in Fig. 3. The unfolding and refolding curves were not superimposed. The CD values after the refolding experiment indicate that only 40% of the protein was refolded at acidic and neutral pH. The overall spectral-shape changes were not observed before unfolding and after the refolding. The CD values at 222 nm after cooling to 4°C were lower than those before heating, even after a month or more at 4°C. Figure 3 shows that the unfolding curves at neutral and acidic pH are significantly different, but the refolding curves at neutral and acidic pH overlap.

Heating Rate Dependence of T_m of Type IV Collagen—Figure 4 shows the heating rate dependence of the CD values at 222 nm of type IV collagen at neutral and acidic pH. The thermal transition curve was markedly influenced by heating rate. At both pH levels, the transition curve shifted to higher temperatures as heating rate increased. To clarify the differences in transition profiles at each pH, the first derivative of the transition curves and calculated T_m are shown in Figs 5 and 6, respectively. Thermal denaturation at neutral pH occurred biphasically; the lower temperature phase had an apparent T_m of ~38°C and the higher temperature phase had an apparent T_m of ~47°C. On the other hand, the thermal denaturation at acidic pH apparently occurred in a single phase with an apparent T_m of ~38°C. The T_m of the first phase at neutral pH and that of the acidic pH were similar.

Kinetic Profiles of Thermal Denaturation of Type IV Collagen—As the thermal denaturation of type IV collagen could not be analysed by the simple equilibrium process, due to the position and shape of the first derivative of the transition curves being affected by heating rate, we analysed it as a rate process. The intensity of CD at 222 nm was monitored as a function of time upon completely native (triple helical) collagen at 4°C, followed by a temperature jump to the desired temperature. The unfolding process occurs in two clearly separate steps: an initial, rapid phase accompanying a steep decrease in amplitude; and a second, slow phase whose amplitude smoothly approaches the end value (Fig. 7). The latter fit best with a first-order rate law. The CD values at time 0 were in agreement regardless of temperature, and the time at which the rapid phase appeared was in agreement until reaching the set temperature. Similar kinetic profiles were observed for

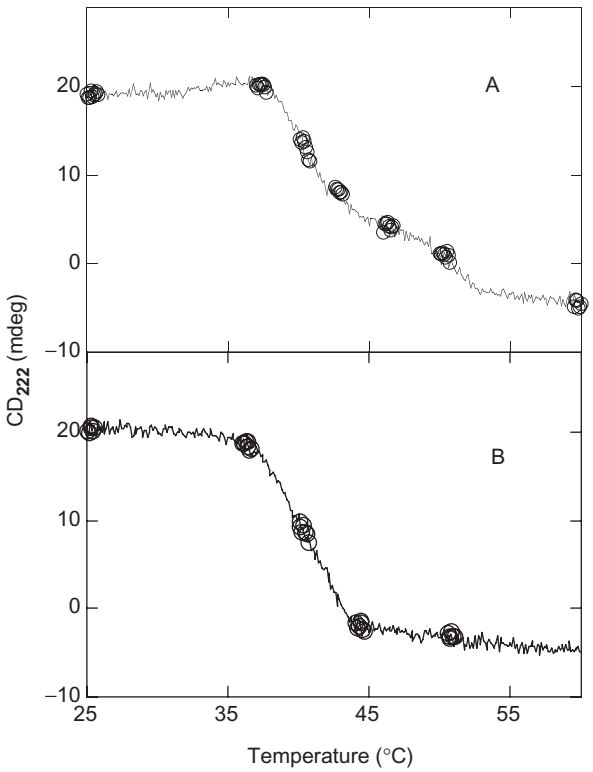

Fig. 2. **Thermal denaturation curve of type IV collagen observed at CD 222 nm at heating rate of 0.5°C min^{-1} (solid line).** Circle shows the CD 222 nm value when UV irradiation was minimized by illuminating samples only during the measuring period. All samples were continuously heated at 0.5°C min^{-1}. (A) and (B) show the data at pH 7.2 and pH 4.3, respectively.

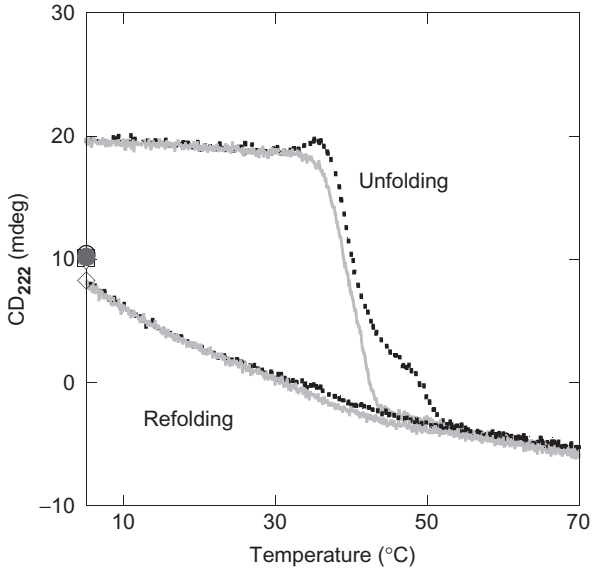

Fig. 3. **Thermal unfolding and refolding curves monitored at 222 nm at temperatures ranging from 5°C to 70°C at neutral pH (pH 7.2, dotted line) and acidic pH (pH 4.3, solid line).** The heating/cooling rate is 0.5°Cmin^{-1}. The concentration of type IV collagen was 0.03% in 50 mM phosphate buffer. Circular dichroism values at various incubation times at 4°C after refolding were as follows: pH4 (△: 1 d, ▽: 3 d, ●: 31 d), pH 7 (○: 1 d, □: 9 d, ◇: 34 d).

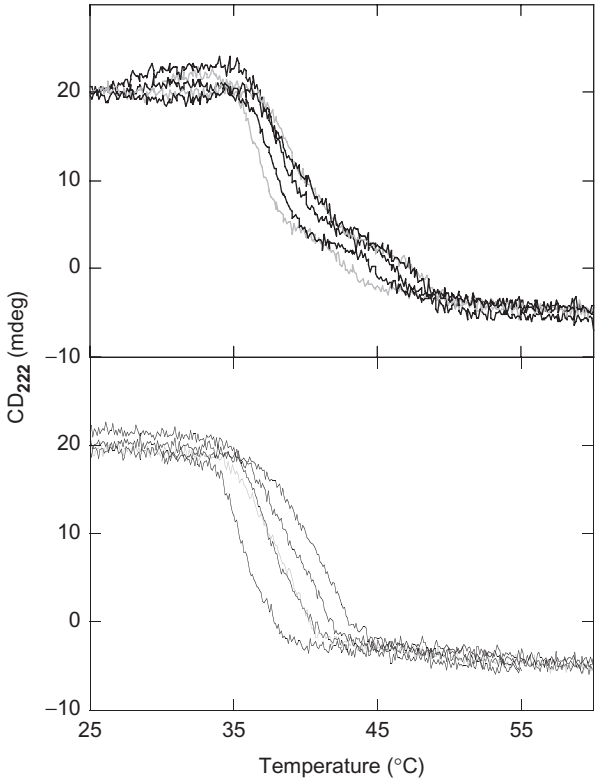

Fig. 4. **Heating rate dependence of thermal denaturation curve of type IV collagen at neutral pH (A) and acidic pH (B).** Concentration of collagen type IV was 0.03% in 50 mM phosphate buffer. The heating rates (°Cmin^{-1}) at neutral pH (pH 7.2) were 0.05, 0.1, 0.5, 0.8 and 1.0, from left to right. The heating rates (°Cmin^{-1}) at acidic pH (pH 4.3) were 0.05, 0.08, 0.1, 0.2, 0.5, 0.8 and 1.0, from left to right.

the refolding processes in the model peptides (*21*). The rate constant at higher temperature phases at pH 7 (Fig. 5A at 42–47°C) could not be analysed, as the rate constant was too rapid.

DISCUSSION

In this experiment, it was clarified that the overall structure of type IV collagen is the same at neutral and acidic pH. To confirm the thermodynamic properties of collagen using spectroscopic methods, it is important that the UV from the light source has no effect on collagen stability, as the thermal stability of collagen is influenced by UV light (*19*, *20*, *23*, *43*, *44*). It has been reported that when samples were irradiated for different periods, new endothermal peaks appeared at ~32°C, in addition to the known peak for collagen at 39°C (*44*). This new peak grew with irradiation time, while the first peak shrank. This indicates that UV light disrupts the native triple helix, and the endothermal peaks are due to the presence of random chains. The primary effect of UV light is to generate free radicals in the water molecules surrounding the collagen molecule, and these radicals react with the collagen, destabilizing it (*44*). At least one of these reactions causes chain scission, which can be detected by SDS page. However, in our CD experiment, no differences between the SDS patterns before and after UV irradiation were observed. This indicates that our UV light source had no effect on native triple helical collagen. It has also been reported that the thermal transition temperature is decreased with UV irradiation, although the shape of the CD spectrum is unchanged. As shown in Fig. 2, the lower shift in T_m by UV irradiation is not observed. From these results, we may conclude that the UV irradiation from the light source in the CD instrument used in this experiment had negligible effect.

It has been proposed that collagen type I unfolding is a reversible (equilibrium) process, as put forward by Flory and Garrett in 1958, and this notion is now generally accepted (*9*, *22*, *38*). On the other hand, some experiments have found that the refolding of collagen is irreversible (*39*, *44–46*). Miles studied the kinetics of collagen denaturation in mammalian lens capsule in great detail by using differential scanning calorimetry (*44*). In that paper, Miles demonstrated that type IV denaturation is an irreversible rate process, following a detailed mathematical treatment of the data (*39*). The importance of NC1 domains in reversible refolding has been reported; removal of NC1 domains by pepsin or dissociation of the hexameric structure by acetic acid leads to loss of refolding activity (*20*). Our data indicate that thermal denaturation of the type IV collagen is irreversible or that refolding is very slow at both acidic and neutral pH.

The thermal denaturation process is significantly different between neutral and acidic pH (Fig. 5),

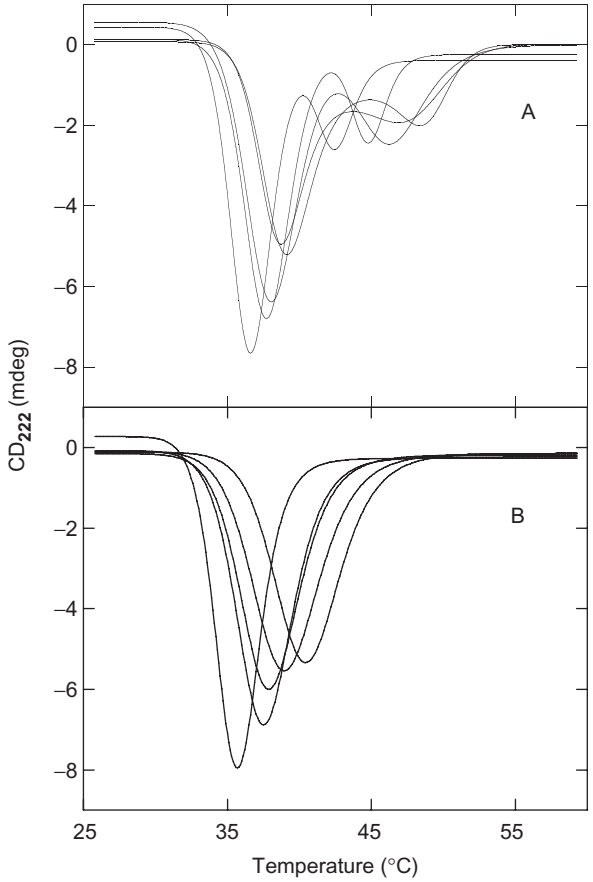

Fig. 5. **First derivative of transition curves in Fig. 4.**

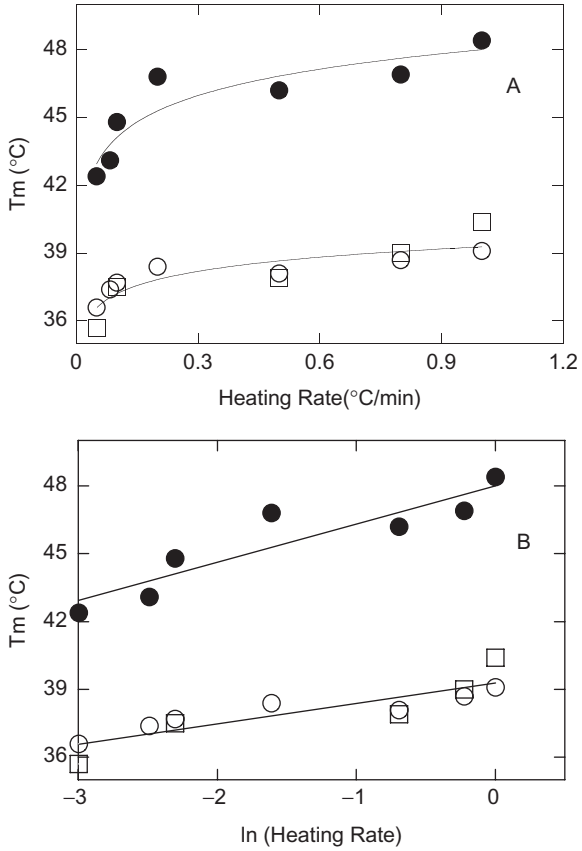

Fig. 6. **Calculated T_m values (temperature of peaks) from Fig. 5 as a function of heating rate (A) and ln (heating rate) (B).** ○: first peak at pH 7.2; ●: second peak at pH 7.2; □: peak at pH 4.3. The line in Fig. 6A and B are the smoothing curve and least squares regression line, respectively.

and tends to be complex, as seen with various types of collagen and model peptides (*22*, *23*, *25–27*, *47–54*). As shown in Fig. 5, thermal denaturation at neutral pH occurs in two phases at all heating rates examined (0.05–1.0°C min^{-1}), while that at acidic pH apparently occurs in a single phase. Thermal denaturation of type IV collagen at neutral pH begins at ~35°C and is completed at ~52°C (Fig. 5). On the other hand, thermal denaturation at acidic pH begins at 35°C and is completed at ~45°C (Fig. 5). The T_m values for the first phase (lower T_m) at neutral pH and at acidic pH are ~38°C, and this phase is not influenced by solution pH. On the other hand, the T_m of the second phase (higher T_m) at neutral pH is ~48°C, and it is more stable than the first phase. At both pH levels, the dependence of observed T_m on heating rate was not linear, even at the lowest heating rate, and could not be extrapolated to equilibrium (Fig. 6A). T_m increased linearly with the logarithm of heating rate for all unfolding steps (first and second phases at neutral pH, and at acidic pH) as shown in Fig. 6B.

Similar linear regression has been reported for the model peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$ (*55*). For this model peptide, the linear correlation was expected to be a first-order rate process. The slope of this linear correlation in the model peptide is large, more than twice that of our type IV collagen. This indicates that the T_m of the thermal denaturation of type IV collagen has low sensitivity to heating rate when compared with that of short triple helical peptides. In the equilibrium process, the endothermic peak observed by DSC is not unaffected by scanning rate. On the other hand, in the rate process, the shape and position of the endothermic peak is dependent on heating rate, and the temperature of the endotherm increases with the logarithm of the scanning rate.

We thus determined the rate constant of thermal denaturation at around the T_m and calculated the activation energy (Table 1). The calculated activation energies were 535 ± 97 and 433 ± 22 kJmol^{-1} at pH 4 and pH 7, respectively (Fig. 8, Table 1). Thus activation energy at acidic pH is consistent with that at the lower temperature phase at neutral pH, within experimental error. Simplified models should, however, include the essentials of real processes and should describe experimental observations in a quantitative manner. The collagen triple helix must undergo major conformational changes in integrin adhesion upon binding and the cell adhesion site must be the first part of molecule to lose its native structure (*21*). The integrin adhesion region, which has a lower triple helical stability, is assigned to the first phase with a T_m of 38°C (*21*). In addition, type IV collagen has several imperfections in the $(Gly\text{-}X\text{-}Y)_n$ repeat necessary for the collagen triple helix (*28*, *29*).

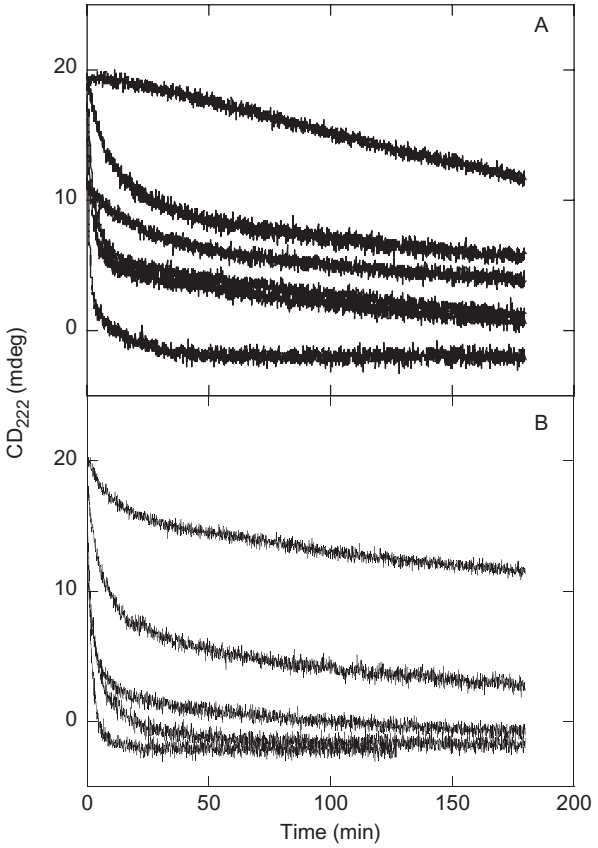

Fig. 7. **Kinetic profiles of type IV collagen at pH 7.2 (A) and pH 4.3 (B).** The intensity at CD 222 nm was monitored as a function of time upon complete native (triple helical) collagen at 4°C, followed by an increase to the desired temperature. The temperature (°C) at neutral pH (pH 7.2) were 39.0, 41.0, 41.8, 43.9 and 45.1, from top to bottom. The temperature (°C) at acidic pH (pH 4.3) were37.0, 38.1, 40.1, and 42.2, from top to bottom.

Table 1. **Rate constants (*k*) at various temperature (*T*) and activation energies for unfolding of type IV collagen at neutral and acidic pH.**

pH 7.2		pH 4.3	
T/°C	$k(10^{-2}\,\mathrm{min}^{-1})$	T/°C	$k(10^{-2}\,\mathrm{min}^{-1})$
38.0	0.866	37.0	0.588
39.0	0.473	38.1	1.27
41.0	0.602	40.1	1.26
41.8	0.784	41.1	5.15
43.9	2.51	42.2	10.5
Ea/kJmol^{-1}		Ea/kJmol^{-1}	
433 ± 22		535 ± 97	

This region and imino-poor sites are thought to be less stable than typical $(\text{Gly-X-Y})_n$ repeats (*56*). The regular triple-helical (Gly-Pro-Hyp) repeats of model type IV collagen peptide are assigned to the second peak with a T_m of 44°C (*21*). Differing stabilities of different regions in type IV collagen have also been reported at neutral pH (*20*, *56*, *57*).

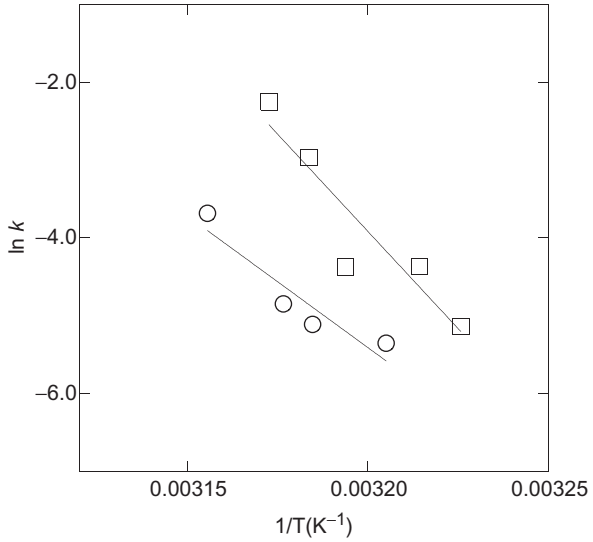

Fig. 8. **Arrhenius plots at pH 7.2 (○) and pH 4.3 (□).** The rate constants used in this plot were calculated from the time course in Fig. 7.

Based on the present results, we can conclude that the thermal denaturation profile of type IV collagen is as follows: (i) at neutral pH, the thermal stability of type IV collagen can be divided into two regions, a stable regular triple-helical (Gly-Pro-Hyp) repeat region (T_m, ~48°C) and an unstable imperfect $(\text{Gly-X-Y})_n$ repeat, near imino-poor sites and integrin-binding regions (T_m, ~37°C); and (ii) at acidic pH, thermal denaturation occurs in a single phase and stability is the same as that of the unstable first phase observed at neutral pH. The unstable and loosely packed regions, such as the integrin-binding region, near imino-poor sites and imperfect $(\text{Gly-X-Y})_n$ repeats, are spread over the whole molecule, and thus the packing of the triple helix loosens at acidic pH. The thermal denaturation of type IV collagen cannot be calculated by the simple equilibrium process, and thus we analysed it as a rate process.

In conclusion, the triple helix of type IV collagen under pathological conditions (acidic pH) is expected to be loosely packed and thermally unstable when compared to normal conditions (neutral pH). The stability at neutral pH is due to the following regions: the stable regular triple-helical (Gly-Pro-Hyp) repeat regions; and the unstable, imperfect $(\text{Gly-X-Y})_n$ repeats, near imino-poor sites and flexible integrin-binding regions (*58–60*). Generally, molecular flexibility is important for molecular interactions, molecular recognition and fibril formation. Therefore, the structural changes in type IV collagen caused by changes in tissue pH may have various effects on pathologies accompanied by degradation of the basement membrane.

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