Disorder of the Structure of Collagen

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INTRODUCTION

Aggregational precipitate of collagen formed upon the addition of ATP is separated from solution of tropocollagen. The electron micrograph of the precipitate shows that tropocollagen, standing side by side, forms paracrystal-like segments (SLS). The SLS has sequential pattern of about 40 sets of stripes. This shows that collagen molecule is composed of the repetition of two blocks of amino acids, one being "band" region and the other "inter-band" region; the former stainable with phospho-tungstic acid or uranyl acetate abounds in polar amino acids, and the latter unstainable with these stains abounds in non-polar amino acids. This blocked structure was also ascertained by the biochemical analysis. So, it is interesting to know how the two blocks contribute to the process of fiber formation of collagen.

The inter-band region has the secondary structure peculiar to the proline-II type, and this structure is thought to stabilize the over-all conformation of collagen. But this fact cannot explain a variety of the fiber formation of collagen. Judging from the amino acid composition, the band region abounds in the amino acids whose homopolymer (synthesized artificially) tends to form clock-wise α-helix rather than anti-clock-wise helix of collagen type. Therefore a balance of the forces must exist between the band region and the inter-band region. This equilibrium of detailed balanced force will sensitively respond to the change in ionic circumstances of the solution and temperature.

Recently, the heat absorption of acid-extracted rat skin tropocollagen was studied by Privalov et al.1 Their calorimetric measurements were carried out under two acidic conditions; one in acetic acid (pH 3.5), salt-free, and the other in acetic acid (pH 3.5) plus 0.1 M NaCl. In the former solution, spectrum of heat absorption showed only a single peak at 42°C in correspondence with a decrease in helical content, whereas the latter showed two peaks at 32°C and 38°C. However, the decrease in optical rotation due to melting of helical structure appeared only at 38°C.
The peak of heat absorption at 32°C was thus explained in terms of change in the intra-
molecular conformation or the interaction of side-chains with solvent. They
 termed “the first stage transition” of collagen structure. Many structural factors
which may contribute to the first stage transition are conceivable. One of them
is a local collapse of the structure of polar region of tropocollagen, the conformation
of which is unstable as we suggested above. To test whether this is the case or
not, we conjugated a fluorescent dye, fluorescein isothiocyanate (FITC), to the polar
region of collagen, and the fluorescence depolarization was measured, as a function
of the temperature with expectation that a possible local structural change in polar
region may be detected by the micro-Brownian motion of the conjugated dye. In
parallel with this, calorimetry was also carried out.

MATERIALS

Rabbit skin and Sprague-Dawley rat skin are cut into chips and homogenized. It
was washed with 1 M NaCl solution three times and with doubly distilled water.
Extraction of tropocollagen was done in acetic acid solution (pH 3.5) for a day in
cold room. The rest was removed with centrifugation at 105 x 10^3 g for 3 hr. Collagen
solution was dialyzed against a large volume of 0.5 M NaCl solution in
cold room. During dialysis, tropocollagen was precipitated and was collected by
centrifugation at 105 x 10^3 g for 30 min, and was dissolved again in acetic acid so-
lution, pH 3.5. For purification, this procedure was repeated 5-6 times until the
optical density at 280 nm per unit concentration became constant, proving the removal
of contaminant proteins.

To obtain the salt solution of tropocollagen, dialysis was performed against
acetic acid solution containing an appropriate salt concentration. Then the last
centrifugation was carried out to remove aggregates.

To obtain the alkaline solution of tropocollagen (pH 9.0), the acetic acid so-
lution of purified rabbit skin collagen was dialyzed against doubly distilled water
for a day, changing the water several times, and then was dialyzed against 0.5 M
carbonate buffer solution (pH 9.0) for two days, changing the solvent. Finally,
centrifugation was carried out to remove the aggregates.

For each preparation of collagen, the last centrifugation was carried out at
268 x 10^3 g for 4 hr, and one third of the solution was collected from the cell.
All the procedures were carried out at 4°C—10°C, and the samples were stocked
at 4°C. We found the rabbit skin collagen was soluble at pH 9.0 (0.5 M carbonate
buffer) (Fig. 1). Several dyes were attempted to conjugate with collagen under
various solvent conditions. However, the best conjugation was obtained when FITC
was bound to collagen under the above-mentioned condition.

The rabbit skin tropocollagen under this condition was monodisperse at 7.5°C and 12.5°C, judging from Schlieren-pattern of the analytical centrifugation.

**Conjugation of FITC to tropocollagen**

We mixed 0.1 mg of dye (FITC; $\varepsilon_493 = 8 \times 10^4$) into 10 ml of 0.5% rabbit skin tropocollagen dissolved in 0.5 M carbonate buffer (pH 9.0) and stirred overnight in cold room. Passing the solution through a column of Sephadex G-25 (about 4 cm x 30 cm), we separated the collagen-dye conjugates from the unreacted dye. The molar ratio of bound FITC/collagen was about 5. Denaturation of collagen upon the conjugation of dye was not found, judging from the appearance of precipitate which was formed when the conjugate was dialyzed against salt solution. This was also evident in the electron micrograph of the precipitate upon addition of ATP. Substantially, degree of optical rotation was identical with that of pure tropocollagen.

**Determination of concentration of collagen**

The nitrogen content of the collagen solution was determined by Kjeldahl method,
and N-contents of both rabbit skin collagen and rat skin collagen were approximated to 19/100 (w/w) = (N/collagen), according to the amino acid compositions of rabbit skin collagen \(^2\) and rat skin collagen \(^3\).

**Fluorescence measurement**

HITACHI MPF-2A fluorescence spectro-photometer was used for fluorescence measurement, and the rate of raising the temperature was 1°C/5~8 min. After fixing the temperature for about 10-15 min, we measured the fluorescence intensity and polarization about 10 times at a particular fixing temperature, spending about 20~25 min. The final result was obtained by averaging them. The slit widths of the excitation (λ = 493 mμ) and those of the emission (λ = 517 mμ) were both 4 mμ.

The degree of fluorescence polarization is defined as:

\[
P = \frac{(I_u - G_h)}{(I_u + G_h)}
\]

The meaning of these signs is explained in detail by Chen et al.\(^4\) The rotational mobility of the conjugated dye can be estimated from the mean rotational relaxation time \(\rho\), which is determined by the Perrin equation\(^5\):

\[
\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right) \left(1 + \frac{\tau}{\rho}\right)
\]

where \(\rho\) is given from Einstein's equation as

\[
\rho = \frac{3v\eta}{kT}.
\]

\(P_0\) is the limiting value of \(P\), of which the absolute temperature \(T\) becomes zero (namely, without depolarization due to Brownian motion), and \(\tau\) is the mean life time of the excited state of fluorescent dye. The linear relation between \(1/P\) and \(T/\eta\) is satisfied by a few proteins \(^6\). When this linear law is followed, the conjugates may be characterized by the quantity \(\beta\)\(^7\):

\[
\beta = \text{slope} / \left(1/P_0 - 1/3\right) = 3\eta\tau / \rho T.
\]

If the life time of the excited state of conjugates is the same, the quantity \(\beta\) is proportional to the mean rotational relaxation time.

**Measurement of optical rotation**

In all measurements of optical rotation, we changed the temperature from about 5°C to about 50°C, and the rate of raising the temperature was about 1°C/5~6 min. After fixing the temperature, we measured the optical rotation at 313 mμ, spending about 1~2 min.

**Calorimetry**

In the experiments where the rate of raising the temperature was 2°C/hr or 4°C/hr, Calvet micro-calorimeter of the standard model MS-70\(^8\) was used in a scanning fashion. In case of the rate 60°C/hr, we used the differential thermal analysis\(^9\) technique. As it takes about a week to carry out one experiment by the
Calvet micro-calorimeter, we used the freshly prepared sample of collagen for each calorimetry, for fear of the denaturation of collagen during storage for several days.

RESULTS AND DISCUSSION

1) *Rabbit skin tropocollagen in the solution of carbonate buffer (pH 9.0)*

In Fig. 2, we see three transitions of the fluorescence polarization at 10°C,

![Graph showing fluorescence intensity and polarization](image)

![Graph showing fluorescence intensity and polarization](image)

Fig. 2 The fluorescence intensity and polarization of FITC conjugated to rabbit skin collagen

- : fluorescence intensity, o : fluorescence polarization in 0.5 M carbonate buffer (pH 9.0)

Concentration of the collagen was 0.01%.

21°C and 41°C. Generally, the lifetime of conjugated dye is nearly proportional to the fluorescent intensity of dye. Fluorescent intensity decreased smoothly in the temperature range from 4°C to 50°C by about 20%, and there was no special change in correspondence with the transition of polarization at 10°C, 21°C and 41°C. Therefore, we can take $\beta$ as a measure of the rotational mobility of conjugated dye.

The slope and the $1/P_0$ in Fig. 2 are: (a) $0.1 \times 10^{-4}$, 4.8; (b) $4.1 \times 10^{-3}$, 5.2; (c) $1.8 \times 10^{-2}$, 2.1; (d) $1.5 \times 10^{-2}$, 4.5; respectively. For the value of (d), the actual slope will be in the range of $1.0 \times 10^{-2} \sim 2.0 \times 10^{-2}$ due to a large fluctuation of the $1/P$ value in this temperature range. The values of $\beta$ calculated from these figures are: (a) $2.2 \times 10^{-3}$, (b) $4.1 \times 10^{-4}$, (c) $1.1 \times 10^{-2}$ and (d) $2.3 \times 10^{-3}$. The
The large difference between (b) and (c) indicates that the rotational mobility of the conjugated dye increased at 21°C. From Fig. 3 (D), there is no decrease of the helix content at this temperature, so that the increase in the rotational mobility may be caused from a local collapse of collagen, while the increase in $\beta'$ between (c) and (d) is caused from the melting of helical structure at 41°C (Fig. 3 (D)).
ment of fluorescent polarization, the transition in most of the experiments was rather clear. These different results would come from a slight difference in the rate of raising the temperature, as is suggested in the following calorimetry.

In calorimetry, when we changed the temperature at a rate faster than 60°C/hr from 4°C to 35°C, and then at the rate of 2°C/hr from 35°C to 50°C, the heat absorption for one gram of collagen (at 41°C) was detected to be 22 ± 2 cal (Fig. 3(A)). However, at the rate of 2°C/hr from 15°C to 50°C, we found 1~5 cal/g as the heat absorption at 41°C (Fig. 3(B)), and in the case of 4°C/hr, we found 7~11 cal/g at the same temperature (Fig. 3(C)). If the melted structures of collagen above 42°C of (A), (B) and (C) are the same, there must be a certain amount of heat absorption under 35°C, because the initial states of (A), (B) and (C) at 4°C are the same. So, in the course of the temperature change from 4°C to 50°C, they must give the same quantity of total heat absorption from (A), (B) and (C); the total quantity of heat absorption must be the same, independent of the rate of raising the temperature.

Judging from Fig. 2, this possible heat adsorption would appear at 20°C ~ 35°C. But so far, we could not yet ascertain this, on account of a large noise from the calorimeter in the temperature range from 20°C to 35°C.

Fig. 4 shows the heat absorption at the rate of raising the temperature of about 60°C/hr. As is expected from the reasoning mentioned above, a single peak at 41°C appeared. Since the technique was tentative only to detect the position of heat absorption, we could not calculate the exact quantity of this heat absorption.

![Fig. 4](image_url)

**Fig. 4** Differential thermal analysis thermogram of rabbit skin collagen in 0.5 M carbonate buffer solution (pH 9.0)

The rate of raising the temperature was about 60°C/hr; concentration of collagen was 0.5%.
2) *Rabbit skin tropocollagen in acetic acid solution (pH 3.5), salt-free, or plus 0.1 M NaCl*

So far, we have no datum of calorimetry of these collagens below 35°C and for the rate of raising the temperature except 2 °C/hr. In acetic acid (pH 3.5), salt-free, we have heat absorption at 41°C (Fig. 5 (A)) and in acetic acid (pH 3.5) plus 0.1 M NaCl, we have one at 38°C (Fig. 5 (B)). These are in correspondence with the decreases in optical rotation.

The heat absorptions of (A) and (B) of Fig. 5 are both less than 5 cal/g of collagen. If the total heat absorption from 4°C to 50°C is about 20 cal/g of collagen, heat absorption of about 15 cal/g must exist below 35°C, with the same reasoning mentioned above for alkaline solution. A large rate of raising the temperature may increase the quantity of heat absorption at 41°C for (A) and at 38°C for (B).

The fact that the melting temperature of collagen structure in these two solvents determined by optical rotation are the same implies that the stability of the helical structure of collagen is not weakened in this alkaline solution.

![Fig. 5](image)

Fig. 5 The optical rotation and the heat heat absorption of rabbit skin collagen in acidic solution

(I)'s of (A)' and (B) are the degrees of the optical rotation as a function of temperature, and (2)'s of (A) and (B) are the heat absorptions, of which the rates of raising the temperature were both 2°C/hr.

(A) : the rabbit skin tropocollagen in acetic acid (pH 3.5), salt-free; (B) : in acetic acid (pH 3.5) plus 0.1 M NaCl.
3) Rat skin tropocollagen in acetic acid solution (pH 3.5), salt-free, or plus 0.1 M NaCl

As a large noise could not be eliminated effectively in calorimetry of these collagens below 35°C, we have no datum for the rate of raising the temperature except for 2°C/hr. The calorimetry was done in the same procedure as in the rabbit skin collagen. No heat absorption was observed above 35°C in spite of a decrease in helix content of collagen at 41°C (Fig. 6 (A)) and at 38°C (Fig. 6 (B)). Privalov et al. got a single peak of heat absorption at 41°C in the salt-free solution and two peaks at 32°C and 38°C in salt solution. The difference found in the results of ours and Privalov et al.'s would come from the different rates of raising the temperature: ours, 2°C/hr, and theirs, 12°C/hr.

Fig. 6 The optical rotation and the heat absorption of rat skin collagen in acidic solution. (1)'s of (A) and (B) are the degrees of the optical rotation as a function of temperature, and (2)'s of (A) and (B) are the heat absorptions, of which the rates of raising the temperature were both 2°C/hr. (A): the rat skin tropocollagen in acetic acid (pH 3.5), salt-free; (B): in acetic acid (pH 3.5) plus 0.1 M NaCl.
However, as the helix-coil transition took place at 33°C in salt solution and at 42°C in salt-free solution, lack of the heat absorption at these temperatures must be explained. One of the reasons may be the difference in the rate of raising the temperature between the calorimetry and the measurement of optical rotation. Heat absorption must exist below 35°C, being of the order of at least 17 cal/g of collagen as Privalov et al. showed.

Taking various kinds of collagen, Privalov et al. also showed that both the melting temperature and the amount of heat absorbed linearly increased with the amount of imino acid residues. The imino acid content of rabbit skin collagen is 284 per 1000 residues of the total amino acids \(^1\). From this value, we estimated the transition temperature as 56°C, and the quantity of heat absorption as 23.5 cal/g of collagen. Heat absorption of 22 ± 2 cal/g of rabbit skin collagen in carbonate buffer is likely reasonable. The transition temperatures of this collagen in carbonate buffer and in acetic acid (salt-free) are both 41°C, which are appreciably lower than the expected values.

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コラーゲン分子の二次構造のくずれ

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可溶性collagenの会合沈殿の電子顕微鏡観察によれば、collagen分子は、極性アミノ酸に富む「band」領域と、非極性アミノ酸に富む「inter-band」領域とが、交互にくり返した構造をとっていることがわかる。Collagen分子の3次元的構造は、強い左巻きの3本のhelixがゆるく右巻きに織り合わさっていると考えられているが、band領域はそれの合成homopolymerのhelixは右巻き、inter-band領域の左巻きが安定であるところのアミノ酸によって、それぞれ占められている。従って、band領域の右巻きとinter-band領域の左巻きとの間には、一定の力のバランスが存在し、この力のバランスは、外部の塩の条件や濃度などの変化に対して敏銳に反応するものと思われる。

こういったことに対する手がかりを得るために、rabbit skin tropocollagenの極性部—band領域—に蛍光色素FITCを付けて、温度変化に対するその蛍光の強度、偏光の解消の仕方などを調べた。これと平行して、熱測定も行なった。

アルカリ条件下でのrabbit skin tropocollagen＋FITC conjugateの蛍光強度は、温度変化に対しスムーズに変化したが、偏光度は10°C, 21°C, 41°C附近で大きく減少した。一方ORの測定結果は、41°Cでその減少をみたのみであった。従って、21°Cにおける偏光度の変化は、band領域の構造をくずれを示していると考えられる。さらに偏光度測定の結果は、12°C～41°Cの領域にても、徐々に極性部分の内部構造がくずれていく事は示している。

また熱測定からは、温度上昇の速度を遅くするにつれて、41°Cのmeltingに相当する熱吸収量が減少して行くことが分かった。Meltした後のcollagenの構造は温度上昇の速度によらないと考えられるので、この熱量の減少は、未測定の温度領域（35°C以下）にて補償されねばならない。

イミノ酸の含量と熱安定性との関係は、Privalovらの推論に同致しなかったが、meltingにおける熱吸収量はreasonableであった。

また、rat skin tropocollagenの酸性条件下で、ORの減少する温度における熱吸収が認められなかった事実は、ORと熱測定の間の温度上昇のprocessの差異が主因と思われるが、上のrabbitの場合と同じ理由で、35°C以下に適当な熱の吸収があるものと考えられる。

いずれにしても、温度上昇の速度が小さい場合には、band領域の局所的な構造のくずれを問題にしなければならないようである。
東條 英氏
蛻光色素がコラーゲン分子の polar な部分につくという事の論拠を伺います。

由良 正信氏
Fluorescein isothiocyanate (FITC) は

\[
\text{HO} \quad \begin{array}{c}
\text{O} \\
\text{SCN} \quad \text{COOH}
\end{array}
\]

という構造を有しており、この色素は、側鎖であるシアン基 (-NCS) によって、タンパク質の主として遊離アミノ基と

フルオレッセイン -N=C + N-タンパク質

\[
\begin{array}{c}
\text{S} \\
\text{H} \\
\text{H}
\end{array}
\]

のようにして、固い化学結合をすると言われております。

コラーゲンに関しては、FITC は、例えばアルプミン・トロポミオシン・トロポニン・などどのタンパク質と比較する限りでは、非常に付着しにくく、従って我々は、コラーゲンに付着しているFITC は、ほとんどはリジンなどの遊離アミノ基に付着しているであろう、と予想しております。しかしながら、その事に対する解析は何ら行なっておりません。