Effect of pH on Thermal Stability of Collagen in the Dispersed and Aggregated States

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Thermal stabilities of mature insoluble collagen, salt-precipitated fibrils of acid-soluble collagen and acid-soluble collagen in solution were compared as a function of acid pH. Both insoluble and precipitated collagens showed large parallel destabilization with decrease in pH, whereas the intrinsic stability of individual collagen molecules in dilute solution was comparatively unaffected.

In common with proteins generally, the tertiary structure of collagen molecules in solution exhibits heat-lability resulting in intramolecular structural 'melting' over a narrow temperature range. The temperature at the transition midpoint (or 'melting temperature'), as measured by polarimetry or viscosity, is dependent on various internal and environmental factors such as the collagen source and pyrrolidine residue content, the presence of structural perturbants such as polar organic solvents and neutral salts (reviewed by von Hippel, 1967) and the pH of the aqueous solvent medium (Burge & Hynes, 1959; Dick & Nordwig, 1966; Woodlock & Harrap, 1968; Hayashi & Nagai, 1973).

Further organization of the collagen molecular units within native fibril structure *in vivo* results in substantially enhanced stability due to the superimposed molecular interactions as well as the progressive formation of a network of insolubilizing covalent cross-links. Stability changes in fibrils in response to variations in the aqueous environment differ in a number of respects from that in solution (Veis, 1967; Ciferri, 1971); other instances of such differential response are evident from comparison of the effects of polar organic solvents and neutral salts on the excess of stability in fibrils and on collagen structure in solution (Bianchi *et al.*, 1967; Russell & Cooper, 1971; Russell, 1973, 1974).

In the present communication, a study of the comparative pH-dependence of thermal stability in acid-soluble collagen in dilute solution, in salt-precipitated fibrils derived from the same soluble collagen preparation and in mature insoluble collagen is reported.

Materials

Acid-soluble calf skin collagen. This was extracted and purified by the methods previously described after removal of the neutral-salt-soluble fraction (Cooper & Davidson, 1965).

Precipitated acid-soluble collagen fibrils. Fibrils showing native cross-striation were formed as previously described (Russell, 1973) by mixing equal volumes of a solution of acid-soluble calf skin collagen (2mg/ml) in 0.15M-potassium acetate buffer (pH 4.8) and a solution of 0.25M-KCl in 0.15M-potassium acetate buffer of the same pH. Fibrils were allowed to precipitate at ambient temperature (approx. 16°C) for 2h. Examination of the resulting fibrils in the Hitachi transmission electron microscope after staining in 1% (w/v) phosphotungstic acid adjusted to pH 6.3 (Russell, 1973) confirmed the presence of predominantly striated fibrils with native 64nm periodicity.

Intact insoluble collagen. An area of intact fibrous collagen for shrinkage temperature measurements was prepared from limed and acetone-dehydrated sheep skin as previously described (Russell & Cooper, 1971) to give an insoluble collagen matrix conforming to commercial hide powder of approx. 99.6% collagen content by weight [Official Methods of Analysis (1965), Society of Leather Technologists and Chemists, Redbourn].

Reagents. Chemical reagents used were A.R. grade or laboratory-grade materials.

Measurement of thermal-transition temperatures

'Melting' temperature of acid-soluble collagen in solution. 'Melting' curves were measured on a Perkin-Elmer 141 digital polarimeter by using the '30min method' (von Hippel & Wong, 1965) on collagen solutions (1mg/ml) in potassium acetate buffer at various pH values in the range 2.5-5.0 and 0.15M with respect to acetate ion concentration. To minimize dilution, pH adjustment was effected after the freeze-dried collagen had been dissolved at 5°C in 0.15M-acetic acid, by titrating the solution by briefly immersing a KOH pellet with the stirred solution in contact with a pH electrode. The temperature at the

midpoint of the optical-rotation transition was taken as the 'melting point'.

Dissolution temperature of salt-precipitated acid-soluble collagen. Thermal stability of fibrils was examined as previously described (Russell & Cooper, 1971; Russell, 1973) by removing and resuspending a small portion of the freshly precipitated material in approx. 10ml of pH-adjusted acetate buffer. Dilution of the solution carried over on the fibrils was considered sufficiently high to lower the Cl^- ion concentration to a negligible value. The resulting fibril suspension was heated ($0.5\text{--}1^\circ\text{C}/\text{min}$) in a test tube supported in a beaker of water stirred on a magnetic stirrer-hotplate. The suspension was stirred at intervals by means of the thermometer bulb immersed in the test tube. The dissolution temperature of the suspension as gelatin when illuminated from the side and viewed against a dark background was noted. Since the presence of minimal amounts of added halide ions was necessary to keep the precipitated fibrils from spontaneously redissolving at acid pH values, it was not possible to measure directly the thermal stability of fibrils in buffer solution alone. However, virtual thermal-stability values could be obtained by carrying out measurements over a range of added salt concentrations and extrapolating to zero salt concentration.

Shrinkage temperature of insoluble collagen. Samples for shrinkage-temperature measurements were cut from the area of prepared sheep skin collagen in $0.5\text{cm} \times 1\text{cm}$ strips and randomized. The apparatus used consisted essentially of a fixed lower and a movable upper clamp for sample mounting, relative movement of the two clamps being magnified fivefold by a pulley and pointer attachment which also served to keep the sample under slight tension. The clamped sample was immersed in a beaker of buffer solution under examination and the temperature raised at approx. $0.5^\circ\text{C}/\text{min}$. The temperature of incipient length contraction (or 'shrinkage temperature') of the strips exposed to various pH values in 0.15M -potassium acetate was measured [Official Methods of Analysis (1965), Society of Leather Technologists and Chemists, Redbourn].

Mathematical model

Experimental transition temperatures as a function of solution pH were fitted by a least-squares technique, by using a non-linear regression program written for a Hewlett-Packard 9100 B programmable calculator, to a mathematical expression of the following form:

$$T_{D_i} = T_D^0 + \frac{m \cdot 10^{pK - pH_i}}{1 + 10^{pK - pH_i}}$$

T_{D_i} and T_D^0 represent the transition temperatures at pH_i and at a higher pH value at which the available

acidic groups are fully dissociated (i.e. in the isoelectric range, $\text{pH } 6\text{--}8$), m represents the amplitude of T_{D_i} variation, and the ratio $10^{pK - pH_i}/(1 + 10^{pK - pH_i})$ the proportion of acidic groups dissociated.

The model assumes that depression of the thermal transition temperature for collagen both in solution and in fibrillar aggregates relative to that observed under isoelectric conditions is directly proportional to the excess of cationic charge resulting from the discharge of acidic groups by protonation at acid pH values. On this basis, the pK value extracted from the experimental data is a measure of the apparent mean dissociation constant for the side-chain carboxyl groups of aspartic acid and glutamic acid residues under the experimental conditions adopted.

Results and discussion

Since freshly precipitated collagen fibrils tended to redissolve spontaneously at acid pH, thermal-stability measurements could not be made in the absence of small amounts of added salt to suppress solubility. Typical variation in fibril stability with pH in the presence of KBr in the concentration region above the precipitation point is shown in Fig. 1.

Fig. 1. Typical neutral salt effects on thermal stability of precipitated acid-soluble calf skin collagen at various pH values

The salt used was KBr in pH-adjusted acetic acid-potassium acetate, 0.15M in acetate ions. Broken lines denote back-extrapolated portions of experimental plots extending over the low salt concentration range when spontaneous dissolution of the precipitated fibrils occurred at ambient temperature. $\circ$, $\text{pH } 3.0$; $\triangle$, $\text{pH } 4.0$; ∇ , $\text{pH } 4.5$; $\square$, $\text{pH } 4.7$; $\diamond$, $\text{pH } 6.5$.

Fig. 2. Effect of pH on thermal stability of collagen in solution and in fibrils

Stabilities as reflected in optical-rotation transition temperature of acid-soluble calf skin collagen in solution ($\circ$), dissolution temperature of salt-precipitated striated fibrils from the same acid-soluble collagen source (Δ), and shrinkage temperature of mature insoluble sheep skin collagen ($\square$). All measurements were made in pH-adjusted acetic acid-potassium acetate, 0.15M in acetate ions. Data for precipitated fibrils at zero salt addition (broken line) were obtained by extrapolation of trends in Fig. 1. Parameter estimates corresponding to the generated curves are indicated in parentheses as follows: (T_D , m , pK).

Extrapolation of the experimental trends to zero salt concentration yielded intercepts corresponding effectively to fibril stabilities in the absence of added salt. Gradients of the extrapolated portions of the plots decreased progressively as the pH increased with accompanying shift in precipitation limit to lower concentration, resulting in increased accuracy in extrapolation.

Experimental values for the extrapolated thermal stability of precipitated collagen as a function of pH are shown in Fig. 2 together with the corresponding transition temperatures for acid-soluble collagen in solution and mature insoluble collagen. Values for collagen in solution could not be extended above pH 5 owing to the increased tendency for precipitation to occur when the temperature was raised for stability determination. Graphical trends shown are theoretical trends generated by the mathematical model described by using the least-squares estimates of the parameters T_D , m and pK calculated from the data.

For acid-soluble collagen in solution, there was evidence of further stabilization as the pH was lowered below 3. These values were not included in the data used for parameter estimation. Similar stabilization of collagen at pH 3 in the absence of added salt has been reported in optical-rotation studies on soluble rat tail-tendon collagen by Woodlock & Harrap (1968), but was not apparent in similar viscometric and optical-rotation studies on a variety of soluble collagens reported by other investigators (Burge & Hynes, 1959; Dick & Nordwig, 1966; Bianchi *et al.*, 1967; Hayashi & Nagai, 1973). The reasons for the stability increase in solution at acid pH values are not clear and there did not appear to be any comparable effect for fibrils in the same pH region. With the exception of the deviant values for collagen in solution, the experimental observations for the three collagen types conformed closely to the theoretical model proposed.

A feature of the experimental observations was the comparative insensitivity of collagen in solution to pH variation ($m = 1.7^\circ\text{C}$), indicating that charge effects play a minor role in determining the intrinsic stability of isolated molecules in solution. The situation differed considerably for precipitated and insoluble collagen fibrils, which showed large pH-dependence ($m = 20^\circ\text{C}$). The striking parallel observed between insoluble collagen and precipitated collagen fibrils supports the contention that both macroscopic thermal shrinkage and thermal dissolution of precipitated fibrils are manifestations of the same underlying structural transition. Previous examination of the material in solution at temperatures fractionally above the fibril dissolution temperature yielded specific-rotation values corresponding to gelatin ($[\alpha]_{565}^{25} - 460^\circ$), indicating that fibril dissolution had resulted from a helix-to-coil transition in the material (Russell, 1973).

Similarity in the response of both native fibrils and precipitated fibrils supports the electron-microscopic evidence that the mode of molecular organization is the same in both cases, notwithstanding a limited degree of chemical modification such as conversion of arginine into ornithine residues and hydrolysis of amide groups of asparagine and glutamine to be expected as a result of alkali pretreatment of the insoluble collagen (Bowes & Kenten, 1948). The uniform excess of stability in insoluble fibrils relative to the precipitated collagen, amounting to some 9°C , appears to be due in part to the presence of covalent intermolecular cross-links responsible for insolubility in the former.

The pK values for the two fibril types were comparable, but slightly lower than the value found for collagen in solution. The values conformed to the range expected for carboxyl-group titration in sheep and bovine collagens and gelatin (3.5–4; Bowes & Kenten, 1948; Veis, 1964).

A further feature of the experimental results was the apparent tendency for fibril stability at pH values below 3.5 to fall below that of the individual collagen molecules in solution. Although the reasons underlying this unexpected situation are not clear, it would appear that, whereas complementary charge interactions exert a considerable stabilizing influence in fibrils under isoelectric conditions, conversely, strong charge repulsions developed within fibrils at low pH have a considerable disrupting effect on the intrinsic stability of the component molecules.

The comparatively small effect of pH variation on collagen in solution in the present study may be contrasted with previous reports in which denaturation was measured as a function of pH in the presence of added salts (Burge & Hynes, 1959; Dick & Nordwig, 1966). In their studies, the amplitude of the stability-pH transition was approx. 8°C. Examination of anion effects on collagen stability at acid pH values reveals substantially enhanced lyotropic activity, particularly at low salt concentrations (Bianchi *et al.*, 1967; Woodlock & Harrap, 1968). Lyotropic activity of the halide and thiocyanate ions increases rapidly with increasing acidity, indicating that the effect is potentiated by the excess of cationic charge developed across the molecules in acid solution (Russell, 1974). These observations indicate that, in the presence of lyotropic salts, a large stability-pH amplitude for collagen in solution can be expected. For the weakly lyotropic acetate ion used in the solvent medium in the present study, the relatively small amplitude observed would appear to reflect correctly the intrinsic insensitivity of collagen molecules in solution to charge variation.

The results of the present study are consistent with the generally accepted view of collagen structure in which side-chain groups are radially disposed on the cylindrical surface of the elongated molecule, accessible for interaction with the solvent environment and/or neighbouring molecules (Ramachandran, 1967). The relatively small influence of pH on stability of isolated molecules in solution is consistent with an indirect charge effect involving repulsion between positively charged basic groups on opposite sides of the surface, which would tend to distend the molecule at acid pH. A different situation obtains for the molecular arrays within fibrils in which the multiplicity of direct complementary charge inter-

actions confers substantial additional stability, probably due to the increased rigidity imparted to the polypeptide chains, particularly within the crystalline polar zones. Complementary charge interactions, and hence stability, are maximized under isoelectric conditions and conversely decrease rapidly with discharge of the carboxyl groups at acid pH values.

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