Differential Anion Effects on Thermal Stability of Collagen in the Dispersed and Aggregated States

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The effects of KCNS and KI on thermal transition temperatures of calf skin collagen molecules in dilute acid solution and precipitated collagen fibrils from the same source were compared as a function of salt concentration and pH. The two salts produced qualitatively similar effects on each collagen form, but the response shown by single collagen molecules in dilute solution differed from that observed for molecular aggregates present in native-type fibrils.

Destabilization of protein structures in solution in the presence of certain neutral salts is a well-documented phenomenon, reflected in a decrease in the characteristic thermal transition temperature at which the ordered native conformation is transformed into a random coil form (reviewed by von Hippel & Schleich, 1969). Similar lyotropic effects of neutral salts are apparent in dilute solutions of soluble collagen, reflecting salt-mediated changes in the intrinsic stability of isolated single collagen molecules in solution (reviewed by Harrington & von Hippel, 1961; von Hippel, 1967).

In extending studies of the effects of structural perturbants on collagen in solution to the molecular aggregates present in collagen fibrils, it has been found that response in the soluble and insoluble collagen systems differed markedly. In particular, ethylene glycol, glycerol and propane-1,3-diol stabilized collagen in solution (Harrap, 1969; Hart *et al.*, 1971), but destabilized mature insoluble collagen fibres (Russell & Cooper, 1971) as well as precipitated acid-soluble collagen fibrils (Russell, 1973). Similarly, from comparison of stability trends on either side of the salt precipitation point, it was found that addition of KCl progressively destabilized dissolved acid-soluble collagen, but stabilized the precipitated fibrils formed from the same material (Russell, 1973).

These differential effects on dispersed and aggregated collagens indicate that superimposed molecular interactions in collagen fibrils considerably alter the overall stability and resultant response to environmental perturbants. This distinctive behaviour has been further examined. In the present communication, the effects of two neutral salts, KI and KCNS, both of which are strongly lyotropic toward collagen in solution, on thermal stability at various acid pH values, are reported.

Experimental

Materials. Acid-soluble calf skin collagen was

extracted and purified by the methods previously described after removal of the neutral-salt-soluble fraction (Cooper & Davidson, 1965). Reagents used were laboratory-grade materials.

Electron microscopy of precipitated collagen fibres. Collagen fibrils were precipitated as previously described (Russell, 1973) by mixing equal volumes of a solution of acid-soluble collagen (approx. 2 mg of dry protein/ml) in 0.1 M-potassium acetate buffer (pH 4.8) and a solution of KCl in 0.15 M-potassium acetate buffer. The amount of KCl in the second solution was varied initially to give a series of final precipitating concentrations, ranging from 0.03 to 2.0 M, with concentrations doubling in successive solutions. Fibrils were precipitated at ambient temperature (approx. 16°C) for 2 h before removal of a portion of the precipitate for examination. The fibrils were stained in 1% (w/v) phosphotungstic acid adjusted to pH 6.0–6.3 as previously described (Russell, 1973) and examined in the Hitachi transmission electron microscope. A high proportion of negatively stained striated fibrils showing the characteristic native aggregation mode with 64 nm periodicity was evident in fibrils precipitated in 0.125 M-KCl (final concentration after mixing), with decrease in visible striation in fibrils obtained at higher salt concentrations. Accordingly, in subsequent studies of salt effects on thermal stability, striated fibres newly precipitated in 0.125 M-KCl were used throughout.

'Melting' temperature of acid-soluble collagen in solution. 'Melting' curves were obtained by polarimetry by using the '30 min method' (von Hippel & Wong, 1965) on collagen solutions containing approx. 1 mg of dry protein/ml in potassium acetate buffer at various pH values and salt concentrations within the range of protein solubility. To avoid dilution, pH adjustment was carried out by titrating volumes of stock collagen solution (approx. 2 mg/ml) in 0.15 M-acetic acid with rapid stirring in the cold by briefly immersing solid KOH pellets. Various volumes of similarly pH-adjusted 0.15 M-potassium acetate solu-

tion, 1.0M in neutral salt, were added to 5ml portions of protein solution and the mixture was diluted with further 0.15M-acetate solution to 10ml in each case to give a final protein concentration of approx. 1mg/ml and a range of neutral salt concentrations up to approx. 0.2M at fixed pH. Optical rotation was measured at 365nm and at 589nm for solutions containing higher concentrations of KI where absorbance caused by small amounts of iodine liberated in acid media lowered the transmitted light-intensity.

Dispersion temperature of salt-precipitated acid-soluble collagen. Thermal-stability measurements were made on native striated collagen fibrils in 0.15M-acetate solutions at various neutral salt concentrations and pH values within the range of protein insolubility as previously described (Russell & Cooper, 1971; Russell, 1973). A small portion of collagen fibrils previously precipitated in 0.125M-KCl and 0.15M-potassium acetate buffer (pH4.8), as outlined above, was removed on a spatula and shaken in an excess of the neutral salt-potassium acetate medium under examination. The resultant dilution of the solution within the fibrils was considered sufficient to lower the Cl^- concentration to negligible values. 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 on a magnetic stirrer-hotplate. The temperature of dissolution of the suspension as gelatin when viewed against a dark background was noted.

Results and discussion

The relationship between collagen 'melting' temperatures in solution and the dissolution temperatures of precipitated fibrils has been considered previously (Russell, 1973). Specific-rotation measurements in the clear solutions obtained by heating fibril suspensions to temperatures fractionally above the dissolution temperature yielded values corresponding to that of gelatin ($[\alpha]_{365}^{15}$, 460°). This observation indicates that fibril dissolution was associated with a helix-to-coil transition within the aggregated material, analogous to the 'melting' of collagen helices in dilute solution, as distinct from an increase in protein solubility without accompanying structural change. Moreover, changes in thermal stability of precipitated fibrils have been found to parallel closely variations in the shrinkage temperature of insoluble collagen under similar conditions (Russell & Cooper, 1971).

Variation in transition temperatures for soluble and precipitated acid-soluble collagen with KI and KCNS concentration at three acidic pH values is shown in Fig. 1. With increase in salt concentration, a progressive increase in pH of the medium was observed over the ranges indicated in Fig. 1. This small variation was not considered to contribute significantly to the observed effects.

Fig. 1. Effect of (a) KI and (b) KCNS on thermal stability of dissolved and precipitated acid-soluble calf skin collagen at various pH values

The solvent medium was 0.15M-acetate solution adjusted to various pH values with KOH. Open symbols denote 'melting' temperatures for dilute collagen solutions (approx. 1mg of dry protein/ml); closed symbols denote dissolution temperatures for striated collagen fibrils precipitated as described (see the text). Discontinuities marked by vertical broken lines indicate approximate precipitating salt concentrations. pH values: $\circ$, $\bullet$, 2.7-3.4; $\diamond$, 3.5; $\triangle$, $\blacktriangle$, 4.7-5.0; $\blacksquare$, 6.1-6.6.

At the acid extreme (pH 2.7-3.4), collagen stability in solution decreased rapidly with increasing salt concentration up to the precipitating concentration, which occurred in the region of 0.2M for both salts. Gradients

of the plots decreased with increasing salt concentration, however, indicating a progressive decrease in molar effect with increasing concentration. These observations were qualitatively similar to those of Woodlock & Harrap (1968) for effects of various alkali halides and KCNS at acid pH on 'melting' temperatures of soluble collagen from rat tail tendon.

Qualitatively similar lyotropic effects of lower magnitude were apparent at pH 3.5. At the same time, the precipitating salt concentration, particularly for the CNS^- ion, shifted to lower concentrations. At pH values approaching neutrality, no further observations on soluble collagen were possible owing to spontaneous precipitation at all salt concentrations. Effects on soluble collagen stability were generally larger for the CNS^- than for the I^- anion.

The discontinuities in the experimental curves at approx. 0.2M reflect the apparent thermal-stability increase (10–15°C) when fibrils were precipitated in the native aggregation mode and their stability was compared with that of the soluble form in the region of the precipitation point. The stability increase accompanying precipitation appears analogous to the excess of stability ($27 \pm 3^\circ\text{C}$) apparent when the macroscopic shrinkage temperature of insoluble collagen is compared with the 'melting' temperature of the soluble form in solution. In general terms, this stability increase has been attributed to the energy of interaction between collagen molecules ('crystal energy') superimposed on the internal stabilizing forces operating within the molecules themselves (reviewed by Harrington & von Hippel, 1961).

With further increase in salt concentration in the region above the precipitation point, stability variation in collagen fibrils differed substantially from the trend observed in collagen solutions. At approx. pH 3 both I^- and CNS^- ions produced additional fibril stabilization, which reached a maximum at intermediate salt concentrations, followed by extensive destabilization at higher concentrations. Qualitatively similar behaviour was apparent at higher pH, although the stability maximum increased slightly in height and shifted to lower concentrations. At pH 6, the maximum was obscured and only a monotonic stability decrease with increasing salt concentration was apparent. Quantitatively, the effects were again more pronounced for the CNS^- ion.

The present results resemble those of the 'pseudo phase-transition' studies of Bianchi *et al.* (1967). These investigators, however, used viscometry to examine directly the stability of precipitates resulting from heating soluble rat tail-tendon tropocollagen in the presence of various salts. Under these conditions, considerable variation in aggregation mode is possible, depending on temperature, pH and salt concentration. Nevertheless, for KCNS, similar stability variation was apparent with the occurrence

of a maximum as noted for the uniformly striated fibrils examined in the present study.

The differential response of collagen in the dispersed and aggregated states to salt addition suggests that different factors predominate in the stabilization of the two forms. In the absence of extensive molecular interaction in dilute acidic collagen solutions, only a lyotropic anion effect was apparent. At the low pH extreme examined, when the protein carries a large excess of positive charge, the lyotropic effect appeared to be enhanced by the resultant electrostatic repulsion developed across individual molecules in solution. Conversely, a decrease in cationic charge served to decrease the lyotropic effect. Thus, pH increase in the direction of the isoelectric region decreased the initial gradients of the temperature-concentration plots. In addition, with increasing anion concentration, progressive charge neutralization due to preferential anion binding can also be expected, resulting in a decreasing lyotropic effect, as was evident from the negative curvature of the experimental plots.

The role of preferential anion binding to cationic sites on the protein is supported by the occurrence of a critical salt concentration above which charge repulsion between the molecules in acid solution is sufficiently decreased to permit spontaneous precipitation. Since the excess of cationic charge decreases with rising pH, a shift in precipitation point to lower salt concentrations can be expected, as observed experimentally.

The large excess of stability in precipitated fibrils relative to single molecules in solution and the influence of pH and salt concentration indicate that complementary charge interactions between the aggregated collagen molecules constitute an important factor in stabilization, probably owing to the increased rigidity imposed on the component polypeptide chains. The stabilizing effect on fibril structure of further salt addition at acid pH is also consistent with additional residual charge neutralization by preferential anion binding in fibrils, resulting in enhanced molecular interaction, which reaches a maximum under isoelectric conditions. Anion charge interactions appear to predominate over lyotropic effects in determining fibril stability at intermediate salt concentrations. However, when precipitated fibrils are rendered isoelectric at high salt concentration or by increase in pH, an underlying lyotropic effect on fibrils becomes evident.

Non-specific binding mechanisms, involving anion charge interactions at cationic sites and lyotropic binding, considered to involve polar interactions of anions at peptide bonds (reviewed by von Hippel & Schleich, 1969; von Hippel *et al.*, 1973), would account for the observed similarity in iodide and thiocyanate effects as well as the differential response of collagen in the dispersed and aggregated states.

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