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ON THE THERMAL MELTING AND RECRYSTALLIZATION OF RIBONUCLEASE CRYSTALS AND THE HETEROGENEITY OF RIBONUCLEASE*

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Because of the instability of most proteins at elevated temperatures, studies of the crystallization of proteins have generally been conducted at room temperature or lower, while studies at elevated temperatures have been directed to the question of heat-denaturation. This limitation has generally precluded studies of thermal phase transformations of protein crystals; however, for the relatively few heat-stable proteins, such studies should be possible. We have observed melting and recrystallization for one such protein, bovine pancreatic ribonuclease, and found in the results evidence concerning the heterogeneity of ribonuclease.

Samples of ribonuclease crystals of several different modifications were grown in aqueous alcohol or glycol solutions, as described by KING, MAGDOFF, ADELMAN AND HARKER[1]. The crystals were heated gradually in their mother liquors, and were observed to melt to irregular masses of soft, transparent gel over narrow temperature ranges in the region 50–75° C. Generally, the melting was not instantaneous, but proceeded at a rate dependent on the temperature. The "melting points" determined in this work were generally the temperatures at which five to ten minutes was required for melting. These melting points were generally characteristic of the crystal modification used, and were higher for the densely-packed forms, such as form II, or those containing complexed metals, such as form I, than for other forms. No melting was observed if the mother liquor was replaced by a solution more concentrated in alcohol; however, the crystals lost their birefringence gradually on heating above 70° C.

After melting had been observed, the samples were incubated at 25° C, and in most cases, crystals were observed to grow in the mother liquor above the masses of gel. Such crystals were generally of the same modification as had been used for the melting experiments, with the exception that form VII usually gave form I.

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A few of the samples crystallized only after addition of crushed ribonuclease crystal as seed. In Table I are given the approximate melting points of various modifications of ribonuclease, and the crystal types obtained on recrystallization.

In preparations of ribonuclease crystals by the usual techniques[1], more than one crystal form is occasionally observed within the same test tube, the modifications often not transforming into one another after many months. The forms most commonly observed to coexist are II and III. Considered thermodynamically, two solid protein phases should coexist in contact with mother liquor as a trivariant system*.

TABLE I

Crystal form taken	*Crystallization medium*	*Approximate melting point*	*Form obtained on recrystallization*
I	55% MPD*, 1Ni:RNase pH = 5.89	70°	I
II	50% *tert.*-BuOH	70°	II
III	50% *tert.*-BuOH pH = 5	50°	III
V	70% 1,3-propanediol pH = 7.10	>70° (incomplete)	V
VII	55% MPD, 1Ni:RNase pH > 7	60°	I (VII?)
VIII**	55% MPD, 1-3Cu:RNase pH ~ 5.4	75°	VIII

* MPD = 2-methyl-2,4-pentanediol.

** Form VIII is a crystalline modification of ribonuclease containing complexed copper, and will be described more fully elsewhere.

If the pressure, pH, and percent alcohol are fixed, the system should show invariant behavior, and one crystal phase should transform into the other at a sharply defined temperature. However, such phases are often found to coexist over a wide temperature range. This coexistence might be a metastable condition, or might depend on the heterogeneity of ribonuclease, which was shown in the chromatographic studies of HIRS, MOORE AND STEIN[2]. If heterogeneity is the critical factor, then fractionation of the components of ribonuclease between the solid phases should be expected. Chromatographic analysis of specimens of crystal forms II and III isolated from the same test tube showed essentially the same ratio of components A and B.

Further evidence on this problem was obtained by use of the melting phenomenon. Samples containing forms II and III were heated to temperatures between 50° and 70° C, so that only the crystals of form III melted. The samples, now containing unaltered form II, gel, and mother liquor (50 volume % tertiary butyl alcohol), were incubated at 25° C. It was observed that the crystals of form II did not grow at the expense of the gel, but rather, a new crop of form III crystallized from the mother liquor.

The indication of the melting experiments is that the ribonuclease in form III differed from that in form II in some manner other than the known chromatographic heterogeneity, and that this difference persisted during the phase transitions. Some possible ways in which the ribonuclease might differ in these two crystal forms are by having different molecular configurations, or by binding of trace contaminants.

* There are four components: protein, acid, alcohol, and water, and three phases, and hence three degrees of freedom.

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We have found that many contaminants in 0.001 *M* concentration, or less, induce crystallization in form III under conditions in which form II would otherwise be expected. The list of such effective contaminants is so diverse as to include: Zn^{++}, Cd^{++}, $HgCl_2$, NH_3OH^+, acetone, acetate buffer, *p*-hydrazinobenzoic acid, and others. Since coexistence of forms II and III occurs only sporadically, adventitious contaminants are probably responsible. However, in consideration of the non-specific action of the contaminants, both explanations of coexistence of forms may be true, the contaminants being bound in such a way as to induce a configurational change in the protein.

It is apparent from these results that the thermal stability of ribonuclease observed in acid aqueous solution persists in the presence of organic solvents. The formation of gel in the melting process is probably to be explained by the disruption by thermal agitation of the intermolecular bonds of the protein crystal, without alteration of the intramolecular structure. While the gel phase probably has a greater volume than the crystalline phase, observation of the melting of individual crystals shows that the volume increase is not great, and is probably just sufficient to accommodate the protein molecules in a loose, disordered packing. The observed slow rate of melting is to be expected if the diffusion of solvent into the crystal phase controls the rate of melting. Since the crystal phase has lower free energy than the gel at room temperature, it grows from the solvent at the expense of the gel on incubation; recrystallization within the gel by reorientation of molecules is apparently far less likely. The fact that most of the crystal forms recrystallize in the same form again after melting is a strong indication that the internal configurations of the protein molecules are not disturbed during melting. Further, the fact that crystals of one form, present as seeds, fail to grow from the gel produced by a different form indicates that the differences existing among ribonuclease molecules are not affected by the processes of melting and recrystallization.

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SUMMARY

Various crystalline forms of bovine pancreatic ribonuclease, heated in their mother liquors, melt to form gels at temperatures characteristic of the crystal forms used. On incubation at 25° C, crystals grow again in the samples, usually of the same form as had originally been melted. Indications were obtained of a type of heterogeneity other than that previously reported in the literature for this protein.

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