Effect of Freezing and Thawing on the Conformation of Phosvitin*

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SUMMARY

Phosvitin can undergo a change from unordered conformation to β structure upon freezing and thawing. The transconformation may be identical in terms of its product and of key aspects of its mechanism with the conformational change which may take place, without freezing, if the phosphoprotein has been stripped of most of its anionic character, at pH 2 or less, but the freezing effect can occur with solutions that are more weakly acidic by nearly four orders of magnitude. Nevertheless, the effect is enhanced as the acidity of the solution is increased. It is suppressed by increases in salt or protein concentration. The transconformation appears to be linked with the freezing process. Frozen storage is without appreciable effect. Aggregation is an essential corollary of the transconformation: the monomeric protein species gives no evidence of β structure and the extent of transconformation correlates reasonably well with the extent of disappearance of the monomer. The ordered structure is heat-labile and the effect can also be readily reversed by lowering the acidity, but only if the aggregation process has not been allowed to proceed too far. Otherwise, slow continued aggregation leads to the complete precipitation of fibrous phosvitin even at neutral pH. The essential aspects of this freezing phenomenon are consistent with the hypothesis that, as acid is excluded from the growing ice, the acid "tide" just ahead of the liquid-ice interface causes the transconformation, followed by the trapping of the ordered protein in the rigid ice meshwork.

The conformation of phosvitin changes from a largely disordered structure to one of the β type when the ionization of the phosphomonoester groups of this highly phosphorylated protein is suppressed (1). However, if an aqueous solution of the disordered protein is frozen under certain conditions, subsequent thawing produces a solution which contains phosvitin in a folded conformation. This "structure-making" effect of freezing is of interest to us in the particular context of our concern with aspects of phosvitin structure. But such an effect may also account for a number of protein denaturations, and it may be of some consequence in the consideration of conformational restraints imposed on biological macromolecules by their interaction with a structured environment. Hence, we undertook to explore some of the conditions on which the occurrence of this effect depends. Our observations can be explained in terms of a mechanism which is essentially related to the dynamics of the freezing process.

EXPERIMENTAL PROCEDURE

The preparation of phosvitin and the experimental procedures related to CD measurements were described in an earlier report (1). Details of the freezing and melting procedures are given in an accompanying paper (2).

One or another of two techniques of freezing was usually employed. With the "immersion technique," the solution was in a glass test tube, fully immersed in the freezing bath (ethanol-Dry Ice) and held stationary until temperature equilibrium was attained. With the "linear technique," the solution was poured into a Teflon cylinder, closed at the bottom by a platinum-coated copper base which rested in the freezing bath (ethanol, at a given temperature).

Frozen samples were melted at about 30°C. In certain cases, fractions corresponding to the time course of freezing were collected. This was accomplished in one of three ways. (a) The solution was partially frozen by the immersion technique, the liquid was removed and partially frozen, and so on. (b) The solution was frozen by the linear technique, and the frozen cylindrical block was cut into discs. (c) The solution was completely frozen by the immersion technique, and fractions of the melt were withdrawn as they liquified. Fractions collected during melting of samples which had been frozen by the immersion technique represent equally the time course of melting as well as of freezing: with a sample immersed in a heat sink, or heat source, freezing occurs from the outside inward, in the same direction as melting.

As a measure of the redistribution of a given solute upon freezing, the coefficient $K^*$ has been adopted. It is defined as the ratio of the concentrations of solute in the last and the first frozen halves of a solution. When $K^*$ equals 1, solute is assumed to be distributed uniformly throughout the frozen system (2).

Protein concentration values are based on dry weight. Freezing and thawing, under some conditions, causes protein to precipitate; in such cases concentration was determined by phosphorus analysis (3) of the solution after brief centrifugation.

1 The abbreviation used is: CD, circular dichroism.

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The data presented in this paper refer to protein solutions from which no more than about 10% of the original protein may have been lost by precipitation.

CD measurements (with a Cary model 60 spectropolarimeter equipped with a model 6001 CD accessory) usually followed thawing without delay, and the recorded ellipticity data were converted to values of mean residue ellipticity, $\theta$ (degree $\times cm^2 \times decimole^{-1}$), as before (1).

Sedimentation velocity measurements were made with a Spinco model E ultracentrifuge, with the use of the schlieren optical system and cells with filled Epon or Kel-F centerpieces. Calculations, based on measurements of distances on photographic records with a microcomparator (Nikon Shadowgraph) and of areas under the schlieren patterns (with the use of enlarged tracings) with a planimeter (Gelman), were made as before (4).

The photomicrograph of fibrous phosvitin, in aqueous suspension, was prepared with a Bausch and Lomb basic model microscope, adapted to a Polaroid MP-3 camera, with nominally 100 $\times$ magnification and Polaroid type 55 film.

FIG. 1. Effect of freezing on the CD of phosvitin in salt-free, aqueous solution. The solid line (FROZEN, $pH$ 3.0) represents a solution ($pH$ 3.0, 0.05 mg per ml) that had been subjected to five successive freeze-thaw cycles by the immersion technique. The broken lines serve for comparison. The curve labeled NOT FROZEN, $pH$ 1.8 is reproduced from Reference 1 and illustrates the effect of acid on the CD of phosvitin ($pH$ 1.8, 0.2 mg per ml).

RESULTS

Comparison of Low $pH$ and Freezing as They Affect Phosvitin Conformation—That exposure to low $pH$ ($<2$) brings about a folding of phosvitin into a $\beta$ type structure was described in our earlier report on spectropolarimetrically measured changes which occur upon acidification of phosvitin solutions (1). (In Reference 1, we gave our reasons for this conclusion on the basis of the pertinent literature.) Since freezing affects the CD of phosvitin solutions similarly but at higher $pH$ ($>2$), a comparison of the two effects is useful at the outset of this report. Fig. 1 compares the CD of phosvitin frozen and thawed at $pH$ 3.0, with the CD of unfrozen solutions at $pH$ 3.0 (unordered conformation) and at $pH$ 1.8 ($\beta$ type conformation). The CD of the frozen and thawed solution, shown in the figure, is typical of the transconformation produced by freezing. The maximum at about 194 $\mu m$, readily demonstrable with unfrozen samples at low $pH$, can be seen with solutions at higher $pH$, after freezing, only if a slight turbidity (if formed) is not removed by centrifugation before CD measurement. The minimum at about 215 $\mu m$ is marked in either case. With turbid solutions, it shifts to higher wave lengths (as high as 225 $\mu m$). The similarity of the CD spectra of unfrozen, low $pH$ and frozen, high $pH$ solutions of phosvitin suggests that the nature of the ordered conformation produced in the two cases is the same.

Effect of Freezing Conditions—Storage in the frozen state is without appreciable effect on the CD. The spectra of solutions, frozen at $-70^\circ$ and then kept at about $-5^\circ$, $-20^\circ$, or $-70^\circ$ (for up to 2 days), are indistinguishable from those of solutions thawed without frozen storage. When solutions frozen at $-70^\circ$ were kept at $-2^\circ$ or about $0^\circ$ (where some of the sample melts visibly but the “wet” frozen sample persists as such), slight changes have been encountered but $[\theta]_{195}$ becomes no more positive, in about 24 hours, than $-12 \times 10^3$ degree $\times cm^2 \times decimole^{-1}$. Such changes are hardly significant. In contrast, the magnitude of the freezing effect is clearly a function of the number of freeze-thaw cycles to which the solution has been subjected. This is shown in Fig. 2. Maximal effect (under the given conditions) is attained after 5 to 10 cycles.

The effect is not a consequence of low temperature per se but it is linked with the change in state of aggregation of the system. When a solution is supercooled to about $-5^\circ$, frozen suddenly into a spongy ice mass by seeding with an ice crystal (5), immediately separated into liquid and solid portions, warmed to $30^\circ$,
and the CD of the portions is measured, evidence of transconformation is obtained only with the portion which had solidified.

The temperature to which a given already frozen solution may be cooled appears not to affect the magnitude of the conformational change, but the rate of cooling during freezing is not without influence. With the linear freezing technique, the rate of cooling was varied by varying the temperature of the base of the freezing cell. Base temperatures of about -20°, -40°, and -60° produced completely frozen solutions (25 ml, in a tube of 3 cm diameter) in about 2.0, 1.3, and 0.5 hours, respectively. The corresponding values of \([\theta]\) of these solutions, after melting, were -3, +4, and \(-2 \times 10^3\) degree \(\times \text{cm}^2 \times \text{decimole}^{-1}\). That there appears to be an optimal rate of cooling for the production of the conformational change was indicated also by similar experiments with the immersion technique. It may be noted that when phosvitin solutions were frozen very rapidly (in a matter of seconds), in thin layers, the bulk of the protein was found to be precipitated upon melting.

Reversibility of Freezing Effect—Elevated temperatures can bring about a reversal of the freezing effect. For example, phosvitin solutions (pH 3.0, 0.05 mg per ml), which had been subjected to six freeze-thaw cycles by the immersion technique, were incubated at 30° and 100°, respectively, for one-half hour, and their CD was measured: the values of \([\theta]\) were \(-4, -13,\) and \(-17 \times 10^3\) degree \(\times \text{cm}^2 \times \text{decimole}^{-1}\) for the samples thawed at 30° and at 100°, and for an unfrozen control sample. The heat lability of the ordered structure of phosvitin is of interest because of its implication concerning the manner in which the \(\beta\) type conformation may be stabilized. Compared with the contrasting response to heat of the \(\beta\) structures of polylysine (9) and polyserine (7), the \(\beta\) structure of phosvitin is similar to that of polyserine, both being heat-labile.

The question whether a given conformational change may be reversed is usually of interest, but in this particular case it may be raised for a particular reason. It may be recalled that if the ordered conformation of phosvitin is produced by lowering the pH of its solution, the protein can be returned to the unordered conformation by raising the pH (1). This reversal is rapid and essentially complete if prior exposure to low pH was brief; it is rapid but only partial if the acid treatment was of long duration. This suggests a reaction sequence of at least three stages, occurring probably in a partly or wholly concerted manner. These are protonation, folding, and some secondary change such as molecular aggregation (of which the probability is high with a \(\beta\) structure). The reversibility of an observed over-all change in structure may be a function of the relative extent to which the last of these stages, aggregation, has progressed. Whether reversal upon increasing the pH (favoring the reversal of the protonation stage) would be readily effected, would depend on the relative magnitude of rate and equilibrium constants of the individual reactions through which the molecular aggregate grows.

Freezing and thawing of solutions at pH 3 cannot produce the same structure that is produced, primarily, at pH 1.8. At pH 3, any such ordered structure formed upon freezing would have reverted to the unordered conformation upon melting, by the time the pH could be observed: our experiments would have shown no freezing effect. The observed product of freezing could, however, correspond to the secondary, presumably highly aggregated product of prolonged acid treatment. As we shall show presently, the freezing effect manifests itself, in suitable experiments, in a manner consistent with this view.

Dependence of Freezing Effect on pH—In view of the formation of the ordered structure in unfrozen solutions of high acidity (1), and because of its relevance to the mechanism of the freezing effect (to be discussed later), its response to the variation of pH was tested. Fig. 3 (○) shows that the effect is clearly pH-dependent; it becomes increasingly marked as the pH is lowered. A point of particular interest is that a perceptible transition occurs upon freezing when the acid concentration is about two orders of magnitude lower (at pH 3.5) than that required for the transconformation to occur without freezing (at pH 1.8). (Below pH 2.5, freezing results in extensive precipitation.)

If our speculation is valid that the changes in CD described so far reflect the formation of a structure corresponding to the secondary, irreversible product of the exposure to low pH, then the possibility is given that the primary, reversible structure may also be formed upon freezing and become demonstrable under suitable conditions. We tested this possibility by taking advantage of the earlier finding that the rate at which the transconformation in the direction of the \(\beta\) structure occurs increases as the pH is lowered in the region around pH 2 (1). At about pH 2, the acidity of the solution would be both high enough not to permit reversal of any transconformation that may have already occurred and low enough not to bring about appreciable transconformation as a purely pH effect. Therefore, we studied the pH dependence of the freezing effect in such a way that solutions at different pH values were frozen as usual, but were thawed only after addition of an equal volume of 0.01 N HCl to the frozen sample. In this way, as protein is released from the solid into the melt, it is trapped in a liquid environment (at about pH 2) in which its structure, in whatever conformation, would be relatively stable. Fig. 3 (△—△) shows that, with this technique, perceptible transconformation can be readily shown to occur upon freezing when the acidity of the solution is nearly
4 orders of magnitude lower (at about pH 5.5) than that needed to produce the conformational change without freezing (at pH 1.8). Clearly, this finding, in conjunction with the results of the experiments not involving the “trapping” procedure, shows that freezing produces structures which correspond to both the reversible and the irreversible products of acid treatment. Control experiments in which 0.01 N HCl was added to unfrozen solutions already at low pH, showed no effect without freezing (Fig. 3, - - - ). That the trapping technique establishes the formation of a reversible structure can be shown explicitly as well. When a solution at pH 4 is frozen and then melted, trapped at pH 2, followed immediately by raising the pH of the acidified melt to 3 and measuring its CD, the ellipticity (at 197 nrn) of the final solution is found to be a great deal more negative than it was before the final pH adjustment. In contrast, raising the pH of a melt, derived from a solution frozen and melted at pH 3, was effective in lowering the ellipticity only in a very sluggish fashion. On raising the pH to 4 or 6, only about 25 or pH 3, was effective in lowering the ellipticity only in a very sluggish fashion. On raising the pH to 4 or 6, only about 25 or 50%, respectively, of the ellipticity increase produced upon freezing was reversed and only after long exposure (21 hours) to sluggish fashion. 

Dependence of Freezing Effect on Salt Concentration—The transition of phosvitin to a B structure at pH 1.8, can be prevented completely if the ionic strength is high (0.5 M KCl) (1). Salt concentration about 2 orders of magnitude lower (0.001 M KCl) is sufficient to cause an extensive suppression of the freezing effect (Fig. 4). It is of some interest to note that while salt is very efficient in preventing the freezing effect, it is most inefficient in reversing it once it occurred. For example, if a solution at pH 4 is frozen and then thawed into 0.01 N HCl containing 0.1 M KCl, the resulting melt shows CD indistinguishable from that of a melt which contains no salt.

Molecular Aggregation of Phosvitin—If, as we have already suggested, the over-all observed effect (caused either by high acidity or by freezing) is a result of protonation of protein-bound phosphate, folding of the protonated protein, and aggregation of ordered chains, then we may expect that there is a correspondence between the extent of B structure formation and the loss of monomeric protein. This is shown by representative data in Table I. The reasonably linear nature of the correspondence between the degrees of transconformation and aggregation implies further that the monomeric form of the protein cannot be ordered. This was shown also directly, in an experiment in which, with the use of a separation cell (of the movable partition type), monomeric phosvitin was freed of all detectable aggregates, recovered, and its CD measured. While the unfractinated preparation gave evidence of transconformation having occurred to an extent of about 55% (for the basis of this estimate, see Table I), its monomeric component appeared to be totally disordered.

We have also suggested that the extent of aggregation, in terms of average aggregate size, may determine the degree of loss of reversibility. Unfortunately, this suggested relationship cannot be readily assessed in a quantitative fashion because the aggregates which are evidenced by rapidly sedimenting material in the ultracentrifuge are clearly very large and very polydisperse with respect to size. In a semiquantitative way, it is nevertheless clear from a number of experiments that if the protein preparation is such that the transconformation is readily and extensively reversible, then the sedimentation pattern will show appreciable amounts of relatively small aggregates, $s > 30 S$. In contrast, effectively irreversible transconformation is associated with sedimentation velocity patterns which are inconsistent with any aggregate characterized by $s < 100 S$. Thus, extensive aggregation is not inconsistent per se with ready reversibility. For example, the solutions in Experiments 1 and 2 of Table I showed essentially complete transconformation to the B structure before “quenching” reversed it to the indicated low levels. But in view of the reasonable parallel between trans-

![Fig. 4. Effect of freezing on the CD of phosvitin as a function of salt concentration. Aqueous solutions of phosvitin (pH 4.0, 0.05 mg per ml) containing indicated concentrations of KCl were frozen and thawed by the immersion technique, with equal volumes of 0.01 N HCl added just before thawing. The pH of the melt was about 2.2. A, ellipticity data for frozen and thawed samples; O, ellipticity of unfrozen phosvitin solutions containing given concentrations of KCl (at pH 3). See also legend under Fig. 3.](http://www.jbc.org/Downloaded from)

**Table I**

Comparison of extent of transconformation with extent of aggregation

The extent of transconformation is given on the basis of spectropolarimetric data, assuming the maximal observed value of $[\theta]_{197} (10 \times 10^4 \text{ degree} \times \text{cm}^2 \times \text{decimole}^{-1})$ to represent 100%. The extent of aggregation is calculated by difference, on the basis of the relative decrease of the area under the schlieren peak, corresponding to monomeric phosvitin in sedimentation velocity experiments. Measurements in a given experiment were made concurrently, on aliquots of the same solution. “Process” identifies the manner in which the transconformation was produced. “Quenching” refers to the treatment by which the transconformation reaction was effectively stopped. Since the sedimentation of phosvitin requires moderate ionic strength (4), salt was added to all solutions at a point in the procedure given in the footnotes.

| Experiment number | Process | Quenching | Transconformation | Aggregation |
|-------------------|---------|-----------|------------------|-------------|
| 1<sup>a</sup>     | pH 1.9, 0.5 h | pH 3.4 | 4%               | 5%          |
| 2<sup>b</sup>     | pH 1.9, 2 hours | pH 3.4 | 14%              | 2%          |
| 3<sup>b</sup>     | pH 1.8, 2 hours | none   | 72%              | 70%         |
| 4<sup>c</sup>     | freezing, pH 4 | thawing, pH 2 | 45%             | 82%         |

<sup>a</sup> Phosvitin initially in water (0.5 mg per ml); upon quenching KCl added to 0.1 M.

<sup>b</sup> Phosvitin initially in 0.1 M KCl (0.5 mg per ml).

<sup>c</sup> Phosvitin initially in water (0.5 mg per ml); upon quenching KCl added to 0.05 M.
conformation and aggregation, complete transconformation must have been accompanied by complete loss of the monomeric form. It is of some interest also that in nearly 20 sedimentation analyses of phosvitin solutions, representing widely varying degrees of transconformation, no evidence was obtained for the occurrence of small aggregates (s < 20 S) in appreciable quantities.

A final point on the matter of aggregation may be made in connection with the suggested relationship between irreversibility and aggregate size. A stage may be reached in the postulated sequence of aggregation steps beyond which the further growth of the ordered aggregate would become so importantly a function of the increasing thermodynamic stability of the growing complex that the process could lose all dependence on acidity and continue until some ordered structure precipitated from the solution. Fig. 5 shows that phosvitin can indeed precipitate from nearly neutral solution in a completely fibrous form if it was first treated under conditions suitable for the initiation of β structure formation and aggregation.

Changes in CD and pH of Phosvitin Solution in Course of Freezing—Directly related to the question of the mechanism of the freezing effect, it was of interest to determine how solute (acid and protein) might become redistributed upon freezing. Fig. 6 shows that acid is extensively rejected by the growing ice phase: its concentration is much reduced in fractions frozen early in the process and is correspondingly increased in the last frozen portion of the solution. Protein is not similarly redistributed, as shown by the existence of an isosbestic point at about 209 μm, suggesting invariable protein concentration from fraction to fraction. The family of CD curves shows also that the extent of transconformation increases greatly from the beginning to the end of freezing. Although the direction of change in the extent of the transconformation is the same as that of the acid concentration buildup as freezing progresses, there is no obvious quantitative parallel between these changes. For example, the pH in the three center fractions is nearly constant, but the corresponding CD changes are large.

In view of this lacking parallel, it was of interest to determine whether the presence of acid specifically in the ice phase might be nonobligatory, for whatever reason, for the transconformation

Fig. 5. Photomicrograph of fibrous phosvitin at 100 × magnification. An aqueous solution (0.1 mg per ml) was kept at pH 1.85 for 40 min at room temperature. The optically clear solution was then adjusted to pH 7.7 and allowed to stand for 4 days. At that time, the fibrous precipitate was collected and suspended in water. The filtrate was wholly devoid of protein.

Fig. 6. Changes in CD and pH of phosvitin solution in the course of freezing. A salt-free, aqueous solution of phosvitin (50 ml; 0.05 mg per ml; pH 3.02) was swirled in a freezing bath until a portion of the solution solidified. The remaining liquid was withdrawn (the frozen portion remained immersed in the freezing bath) and frozen partially in the same manner. The process was repeated until all of the original solution became frozen, in six fractions. The volume of the individual fractions varied between 4 and 14 ml. Thawing of each fraction (starting with Fraction 1, the first frozen portion) was done as usual, and it was followed by CD measurement and pH measurement, without delay. The numbers next to the CD curves are the fraction numbers. The control curve (broken line) represents the CD of the original solution before freezing. The pH of the thawed fractions is shown as bars to the left or right of the line at pH 3.02, corresponding to the original pH.
to occur. An experiment was carried out (with the linear freezing technique and the freezing base at \(-20^\circ\)) in which a solution of phosvitin (0.05 mg per ml, pH 3.00) was allowed to freeze, while, by careful manipulation of a thin polyethylene tube mounted on a syringe, increments of solution were withdrawn, at intervals in the course of freezing, from immediately above the ice surface. Since the excluded acid solute must be at its highest concentration just above the liquid-ice interface (8), it was possible to withdraw in this simple manner about 55% of the total acid content of the sample in only about 20% of the total volume. It is noteworthy that the remaining sample, which was allowed to freeze completely and then was cut into three fractions and melted, gave evidence of more extensive transconformation having occurred in all three fractions than did another sample in a parallel experiment in which no acid was removed from the system: the average value of \(\theta_{208}\) (averaged for the three fractions) was about \(+3 \times 10^4\) degree \(\times\) cm\(^2\) \(\times\) decimole\(^{-1}\) and \(-1 \times 10^4\) degree \(\times\) cm\(^2\) \(\times\) decimole\(^{-1}\), respectively, in the two experiments.

**Dependence of Freezing Effect on Protein Concentration**—For reasons of its obvious relevance to any question about aggregation and its known effect on the acid-induced transconformation of phosvitin (1), and also because of its importance in the elucidation of the mechanism of the freezing effect, the nature of the relationship between the freezing effect and protein concentration was investigated. Fig. 7B shows that if phosvitin concentration is varied at pH 3.0 (C), the transconformation becomes increasingly extensive with decreasing concentration. This *prima facie* surprising result is confirmed by experiments done at pH 3.0 with (A) or without (B) trapping of the ordered conformation (at pH 2). It is suggestive of an explanation of this unexpected result that the redistribution of acid becomes increasingly more uneven (as seen by the increasing values of \(K_{\text{IH}}^+\) in Fig. 7A) as the protein concentration is lowered. We shall refer to these findings in the course of the discussion of this freezing phenomenon.

**DISCUSSION**

Understanding of the freezing effect on phosvitin structure may be sought within the context of current concepts of the freezing process and of the frozen state. Some of these we referred to in a related study of certain aspects of solute redistribution in freezing systems (2). The same concepts underlie our approach to the principal questions raised by the transconformation associated with freezing. These questions concern the reaction in terms of its relationship to the time course of freezing, its site within the heterogeneous system, and its mechanism.

The conformation of the protein seems to change while freezing is in progress. Frozen storage, at a variety of temperatures, was seen to be of no further consequence beyond the effect of freezing itself. In marked contrast, variations of the rate of freezing and repetition of freezing affected the transconformation. It might be argued that the transconformation is facilitated, for whatever reason, only within a restricted range of temperatures, hence the apparent association of the reaction with the freezing process could be fortuitous; repeated freezing might produce cumulative exposure of the protein solution to such a crucial temperature range, and appropriate freezing rates could prolong such exposure. However, such an argument would predict a monotonously varying relationship between effectiveness and the magnitude of cooling rates rather than account for the apparent existence of an optimal rate. Nor is it likely that storage at any of the temperatures tested, differing by relatively small increments, could be without significant effect over periods of a day or more, when rapid freezing by itself, in the course of which any restricted temperature range is passed through in a matter of seconds, can be highly effective. The distinction between a phenomenon linked with the freezing process and one associated with the frozen state is not trivial. In the former case, the dynamic aspects of freezing must be operative in producing the freezing effect. In the latter case, it is in the nature of the quasi-stable frozen state where the explanation of the effect must be sought.

Considering potential sites of the reaction within the frozen system, the likelihood that the reaction occurs in the solid phase is extremely small. Molecular motion required for a transconformation-aggregation reaction is not compatible with the rigidity of the ice lattice which, even if capable of incor-

**Fig. 7.** Effect of protein concentration on proton redistribution (A) and protein conformation (B). Results of freezing experiments are shown by solid lines and corresponding control data with unfrozen samples by broken lines. Data in A are average values of three experiments, done at different times. Those in B are typical data obtained in individual experiments. Aqueous, salt-free phosvitin solutions of given concentrations (C, expressed as mg per ml; corresponding molar concentrations are given at the arrows) at pH 3.9 (solid symbols) or 3.0 (open symbols) were frozen and thawed by the immunization technique. In some experiments (A), just before thawing, an equal volume of 0.01 N HCl was added to each frozen sample, the final pH of the melt was 2.2 In these cases. For the definition of \(K_{\text{IH}}^+\), the measure of protein redistribution, see "Experimental Procedure." Conformational change is measured in terms of \(\theta_{208}\) which becomes more positive as transconformation in favor of the \(\beta\) structure occurs (The use of \(\theta_{208}\) instead of the more sensitive \(\theta_{203}\) was convenient in these experiments because it permitted the employment of a smaller variety of cells differing in light path than would have been required for ellipticity measurements further in the ultraviolet region, while covering 2 orders of magnitude of protein concentration.)
porating some molecular species other than water, renders all but photons, electrons, and protons essentially immobile (9–12). An alternative is that the reaction occurs in the liquid phase which persists in frozen solutions above eutectic temperatures, in the form of occluded “puddles” (10). The hypothesis that these are the sites of the reaction must be considered with particular care because a number of our observations are clearly consistent with it, and also because it has been eminently successful in explaining a great variety of reactions in frozen systems (10, 11), including an enzymic one (13). However, the indication that the transconformation may be associated with the freezing process rather than the frozen state and also other observations are either incompatible or difficult to reconcile with the puddle hypothesis.

It is generally assumed that, at a given temperature, puddles are liquid regions of concentrated solution in equilibrium with their frozen environment consisting mostly of crystalline solvent (11). The consequence of freezing is simply a buildup of solute concentration in the liquid phase, as solute is rejected by the solid phase (8). Therefore, it seems reasonable to expect that the essential nature of the response of a reaction to changes in solution composition would be the same, whether the reaction occurred in a homogeneous liquid system or in the liquid phase of a frozen system (11). We may usefully contrast the observed influence of compositional changes on the transconformation with this general expectation.

The extent of the transconformation is enhanced as the initial acid concentration is increased in the solution to be frozen. This effect resembles the effect of acidity on conformation in homogeneous solution (1). However, this relationship between acidity and conformation change should be observable not only in terms of the whole system but also of its individual volume elements. Since the acid content (but not the protein content) of the solution is redistributed upon freezing (see Figs. 6 and 7, and Reference 2), analysis of fractions of a given frozen system should reveal essentially the same relationship between acidity and CD as that shown by a series of solutions of varied initial pH when these are analyzed as the whole melt. This, in fact, is not the case. For example, in the experiment depicted in Fig. 6, Fractions 3, 4, and 5, representing different volume elements of the same frozen system have nearly identical acidities but markedly different CD spectra.

In another experiment, in which acid was removed in increments from the unfrozen portion near the interface while freezing was in progress, the eventually measured extent of the transconformation in the whole melt would be expected to be lower than it would have been without the acid removal, simply because of the over-all decrease in the acid content of the system. Interestingly, the opposite was observed. The transconformation is enhanced if the over-all acidity is lowered in this particular fashion.

The freezing effect on conformation depends also on protein concentration, but in a manner opposite to the concentration dependence noted without freezing; increased protein concentration suppresses the freezing effect (Fig. 7) and enhances the low pH effect (1). This finding is in direct conflict with expectation.

The question can be raised whether the expectation with which these results appear to conflict might not be invalidated by some specific features of the relationship between solute concentrations and the nature of the hypothetical reaction sites, the puddles.

In general, increased solute concentration should cause a greater share of the total volume of the frozen system to be occupied by liquid phase (11). According to the hypothesis, the concentration of a single solute in the puddle remains constant, only the volume share of the puddles (the total puddle volume) rises, as required by equilibrium considerations. With more than one solute, puddle volume and concentrations may change, depending on the relative molar quantities of given solutes (which determine the total puddle volume) and on their solubility under the conditions. Even without the specific illustration of particular consequences of these general considerations, it seems to be clear that any changes in volume, or concentration, or both, produced in the liquid phase of a frozen system must be the result of changes in other thermodynamic variables of the system. All else being equal, the observed conformational changes should be uniquely a function of the relevant concentrations of acid and protein, but not of the manner in which their values may be determined in any given experiment. We cannot cope, therefore, with some of the findings cited above within the restrictions inherent in the puddle hypothesis. Different degrees of conformational change, associated with different regions of the frozen system which are characterized by essentially identical acidity and protein content, or differences in the extent of transconformation, depending on the mode of adjustment of the acidity of the system, appear to fall outside the scope of the hypothesis.

The unexpected nature of the relationship between conformational change and protein concentration could perhaps be accounted for by reasoning ad hoc. For example, while increasing concentration favors transconfiguration in homogeneous solution (1), dilution may favor it in the frozen system because of the drastically elevated viscosity which could prevail in the puddles. It may be noted, however, that the viscosity of phosvitin solutions increases with dilution (as it is typical for polyelectrolytes) (14), and that the effect of added salt, which should lower the viscosity, is inhibition rather than enhancement of the conformation change.

Finding that the solid phase is a most unlikely reaction site, and that the puddle hypothesis seems to fail to accommodate some key observations, one alternative remains. The reaction could occur in the solid-liquid interface region which, for a variety of reasons, appears to have a distinct identity (1b). Since some of our results also suggest that the reaction may be restricted to the duration of the freezing process, we may combine the questions concerning reaction site and mechanism and attempt to relate the experimental observations to the dynamic aspects of the moving liquid-ice interface.

The interface advances into the liquid as the ice phase grows. The crystallizing solvent, leading to exclude solutes, drives a “tide” of solute ahead of the moving interface, maintaining a solute concentration gradient which falls from a very high level at the interface to that of the original solution further in the liquid (8). The gradient is sharp; for practical conditions, it has been calculated that it drops by 2 orders of magnitude over a distance from 0.01 to 1 mm, measured from the interface (8). Since the gradient is an exponential one, it is reasonable to expect that within the first 0.01 mm from the interface, the gradient may pass through several orders of magnitude of solute concentration. It is our view that in the weakly acidic protein solutions, it is an acid tide of very high concentration moving just ahead of the interface, which is responsible for the
transconformation. The efficient exclusion of HCl from growing ice (2) supports this view, but its strength comes from its compatibility with a variety of our specific observations.

Assuming that the most concentrated layer of acid near the interface has a thickness of the order of 0.01 mm (see above), protein molecules would be within this layer for periods of the order of 0.1 to 1 sec in our experiments, sufficiently long for extensive transconformation to occur in view of what we know about the rates of the transconformation reaction at low pH (1). Since the freezing effect becomes perceptible at about pH 5.5 and a very rapid reaction may require a pH of about 1.5, the composition of the interfacial layer would need to reflect at most an increase in the concentration of protons by 4 orders of magnitude, which appears to be a reasonable postulate. Once the acid tide passes the protein, its new environment is greatly depleted of protons but the folded structure is prevented from returning to the disordered conformation by incorporation into the solid phase. Being a macromolecule, its slow drift velocity would not permit it to escape becoming trapped in ice. This is reflected in the nearly homogenous distribution of the protein in the frozen system.

Under conditions similar to those of the transconformation experiments, HCl is redistributed upon freezing most unevenly in the pH region about 4 (2). This fits well with the observation that it is in this pH region where the pH dependence of the transconformation reaction shows its most sensitive response to pH changes (compare Fig. 3, A—A). Below pH 4, acid redistribution becomes more even (implying less efficient exclusion from ice and a less steep gradient, although higher absolute concentrations, ahead of the interface) and the pH sensitivity of the transconformation is lessened. Low concentrations of salt suppress acid exclusion from ice (2) and the transconformation (compare Fig. 4). If, as it has been suggested (2), the salt effect is a reflection of a general solute concentration effect on the efficiency of solute rejection by the ice, then the protein concentration effect may be explicable in the same terms. Indeed, the protein concentration effect on the transconformation is correlated with a corresponding effect on acid redistribution (compare Fig. 7).

The result of the experiment involving the removal of acid from the interface region during freezing becomes predictable by this hypothesis. By the time the acid is removed, it may be envisaged to have already fulfilled its role in the conformational reaction. In addition, since the removal itself has lowered the absolute acid concentration in the liquid, an increase in the efficiency of acid rejection and a steeper acid gradient may result, with an increased over-all extent of the transconformation being the ultimate consequence. Similarly, the observation of an optimal cooling rate is compatible with our hypothesis. Rates that are too fast cause inefficient acid rejection, while rates that are too slow will maintain less steep acid gradients because of the added time available for the excluded acid to diffuse into the liquid further from the interface.

In the particular context of phosvitin structure the implications of this transconformation are of interest in view of the fact that in the native environment of the protein, the egg yolk, it is part of a particulate fraction (16, 17)—implying the possibility of ordered structure, in spite of its apparently disordered conformation in solution (1). Indeed, amphibian yolk platelets, containing amphibian phosvitin, have been shown to have a high degree of crystallinity (18, 19).

The postulated mechanism underlying the conformational change may be of some interest in a more general biological context. Among the great wealth of information on biological effects of freezing (20), there is abundant evidence that freezing can affect protein structure (21–31), including a few reports which suggest conformational change (32–35). In some of these cases, a similar mechanism may operate. But it is also tempting to speculate that in an organized biological setting, relationships may exist between ion fluxes, macromolecular conformation, and the structure of the environment, with perhaps some degree of analogy with the acid transport-phosvitin conformation-water structure relationship dealt with in this paper. In view of the interdependence between macromolecular structure and solvent structure (36, 37), and between macromolecular conformation and macromolecular interactions (38–40), it is even possible that a generalized form of the model may imply an element of reciprocity such that an ion flux may be an effect rather than a cause of conformational changes.

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