Denaturation of α-Chymotrypsin in Frozen Aqueous Solutions

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The denaturation of α-chymotrypsin in frozen solutions was studied by two methods: analysis of thawed solutions for activity toward *N*-acetyltyrosine ethyl ester (rate analysis) and titration of active enzyme with *N-trans*-cinnamoylimidazole (active site analysis). Relative to identical nonfrozen (*i.e.*, supercooled) samples, enzyme solutions of 0.05 *M* NaCl, KBr, $CaCl_2$, ethanol, and dioxane frozen at −10° for 96 hr were not denatured by site analysis. Urea solutions at 0.05 *M* caused essentially the same denaturation by either method of analysis and this denaturation was not reversed in thawed solutions by standing at room temperature. The denaturation rate increased as temperature decreased from −5, −10 to −12°, but was slower at −16° than at −12°. Little denaturation occurred in 0.05 *M* urea frozen to −70°. The enzyme was protected from the action of urea in frozen solutions by 0.15 *M* ethanol or 0.05 *M* $CaCl_2$. Denaturation of α-chymotrypsin in 0.05 *M* urea frozen at −10° was rapid to *ca.* 50% activity in 24 hr but occurred much slower thereafter. The results are generally consistent with a "concentration effect" in the frozen solutions together with a denaturation on thawing (*i.e.*, on dilution).

A major factor involved with reactions in frozen systems is the so-called "concentration effect." Any reaction with higher than first-order kinetics will be accelerated (relative to an otherwise identical supercooled system) when the solvent crystallizes out and leaves the solutes at higher concentrations in the remaining liquid phase. This phenomenon has been illustrated many times with simple reaction types (Pincock, 1969). For these the rate and kinetic form may be completely predicted or treated by a combination of data obtained from the reaction in ordinary solutions with knowledge of the phase relationships in frozen solutions. For example, the reaction of 2-chloroethanol with hydroxyl ion has been demonstrated to be up to 1000 times faster in frozen than in supercooled aqueous solutions (Pincock and Kiovsky, 1966). The observed rate maximum at −5° is predicted from a theoretical combination of temperature effects on the second-order rate constant and the equilibrium phase relationship of ice to solution. As well as rate accelerations and rate–temperature maxima, other observed features of some reactions in frozen systems, such as changes in observed kinetic order and decreased rates in the presence of otherwise inert solutes, are also accounted for by a kinetic treatment of the concentration effect.

With much more complex systems such as enzymes, cells, tissues, and whole organisms, the concentration effect has also been implicated as an important contributor to observed changes during frozen storage (Meryman, 1966; Stowell, 1965). However, with most complex systems the contribution of other factors such as thermal shock, osmotic pressure effects on cells, or mechanical damage by ice formation are not easily separated out. In order to approach a more complete understanding of chemical changes in such complex systems, the effects of freezing various solutions containing α-chymotrypsin has been investigated. Use of a single well-characterized enzyme should simplify the interpretation of results, yet be more closely related to results with complex systems. In principle, the kinetic treatment for reactions in frozen solutions can be applied to distinguish the concentration effect from other possibilities. Since "denaturation" of proteins contributes to changes in quality, taste, and flavor of frozen foods (*cf.* Buttkus, 1972), it is hoped that this approach from simple well-established frozen state reactions to a more complex enzymatic system may be of utility in considerations of effects of frozen storage.

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MATERIALS AND METHODS

α-Chymotrypsin (lots 5610 and 6373, three-times crystallized, salt-free) and *N*-acetyl-L-tyrosine ethyl ester was purchased from Nutritional Biochemicals, Cleveland, Ohio. Reagent grade urea was recrystallized three times from ethanol–water. Guanidine hydrochloride was crystallized from methanol by addition of ether.

Ultraviolet absorption measurements were made with a Zeiss PMQII spectrophotometer equipped with a Sargent recorder model SRC. The sample and reference cell were held at 25 ± 0.5° by a circulating water system. Solutions were equilibrated at this temperature before analysis. Measurements of pH were made with a Radiometer model 4 pH meter and are uncorrected for any salt effects.

The unbuffered α-chymotrypsin test solutions were prepared by adding the appropriate solution to weighed enzyme in a volumetric flask. Enzyme concentrations were usually 0.4–0.6 mg/ml. The individual enzyme solution was drawn into a syringe and divided in equal volumes into glass vials which were then closed. For the frozen samples the vials were rapidly partially frozen in a Dry Ice–acetone bath and then transferred to a constant temperature bath to complete the freezing process. After some initial trials of various freezing methods, the above procedure was chosen for its simplicity. Results did not seem sensitive to the amount of freezing in the initial Dry Ice bath before transferring to the bath for a kinetic run. Samples were thawed at selected intervals by immersion and shaking in a 25° water bath for 3–5 min before analysis as given below. Nonfrozen control samples were identical to those frozen but were supercooled at the temperature of the run. These samples often crystallized during a run, necessitating the use of several samples, such that at least one remained unfrozen. Supercooled samples were treated in a 25° water bath in a manner identical to the frozen samples.

Enzyme assays were carried out by two methods. (A) The hydrolytic activity toward *N*-acetyl-L-tyrosine ethyl ester was determined by the spectroscopic method of Schwart and Takenaka (1955). Weighed *N*-acetyl-L-tyrosine ethyl ester was dissolved in 2.5 ml of ethanol and brought to 50 ml by 0.05 *M* phosphate buffer (pH 6.50). Three milliliters of this 0.001 *M* ester solution at 25° was transferred to a 1-cm quartz cell, and 3 ml of 0.001 *M* *N*-acetyl-L-tyrosine in the same buffer solution was placed in the reference cell. At zero time 20 μl of the enzyme solution to be analyzed was added to the cell by a syringe. The test solution was quickly mixed and replaced in the spectrophotometer. The rate of catalyzed hydrolysis of the

ethyl ester was recorded by absorbance changes at 237 mμ over about 5–6 min. The initial rate of reaction (ΔA/min) was taken as the slope of changing absorbance with time during the first 4 min.

(B) The all-or-none assay of Schonbaum *et al.* (1961) was used (method B). This gives the normality of active enzyme. One milliliter of 0.3 *M* potassium acetate buffer (pH 5.80) was pipetted into a 1-cm quartz cell; 2 ml of enzyme solution was added, followed by 50 μl of *N-trans*-cinnamoylimidazole stock solution. The mixture was rapidly mixed and the absorbance at 335 mμ was recorded against a solution of 2 ml of 0.05 *M* urea solution and 1 ml of 0.3 *M* acetate buffer in the reference cell. The relative activity of enzyme solutions is reported below by: (a) the ratio of initial rate of ester hydrolysis in a sample which had been frozen to the initial rate in a sample which had been supercooled, and (b) the ratio of normality of a sample which had been frozen to the normality of a sample which had been supercooled.

RESULTS AND DISCUSSION

Up to 15 repeated freeze–thaw cycles between −10° and 25° do not affect the activity of α-chymotrypsin (0.5 mg/ml) in unbuffered solutions. These are conditions where the ice structure should impinge on the protein and possibly cause dehydration or promote mechanical distortions. Many enzymes are apparently stable to freezing and thawing although, for example, ribonuclease is inactivated most effectively (to 45% activity) at −9° in buffered solutions in eight freeze–thaw cycles (Belikova *et al.*, 1968; Chilson *et al.*, 1965). This deactivation was shown to occur during the freeze–thaw processes themselves and may occur as the system sweeps through an optimum temperature of maximum denaturation brought about by high buffer concentrations in the liquid part of the frozen system (see below). Direct comparisons of effects of freezing to any denaturation in nonfrozen but highly concentrated solutions was not made for ribonuclease, nor indeed have such comparisons been generally made (Tappell, 1966). With chymotrypsin the effect, in ordinary nonfrozen systems, of high concentrations of some denaturing solutes is known (Martin and Frazier, 1963).

Denaturation in 0.05 *M* Solutions. The two methods of analysis for enzymatic activity used here are complementary in that a partial deactivation of every molecule would give a decreased activity in the tyrosine ethyl ester hydrolysis (method A) but would give full activity by the normality analysis (method B). On the other hand, complete deactivation of a fraction of the enzyme molecules with the remaining fractions at full activity would give a corresponding fractional decrease in activity by both methods of analysis. Comparison of results from the two methods of analysis would then show up any minor changes in hydrolytic activity due perhaps to small but not "lethal" changes in the conformation of enzyme brought about by the freeze-storage-thaw cycle. Table I shows the relative activity (frozen sample/supercooled control sample) for various solutes at 0.05 *M* concentration after being frozen for 24 or 48 hr and thawed for analysis. Many solutes which do not directly denature chymotrypsin at 0.05 *M* concentration (*i.e.*, NaCl, KBr, $CaCl_2$, ethanol, dioxane) still do not have any additional effect on the normality of enzyme after being frozen at −9.5° for 48 hr. However, with NaCl or KBr after 24 hr there was a decrease of about 20% in the activity by the tyrosine ethyl ester analysis in freshly thawed solutions. With NaCl solutions, activity was essentially recovered after standing at room temperature 1 hr. The effect is small and is not increased after 48 hr. Its study was not followed up, but such a reversible decrease in activity may reflect conformational changes brought about by high salt concentrations (about 5 *M* at −9.5°). These changes then decrease the hydrolytic activity but do not change the total number of active sites (normality).

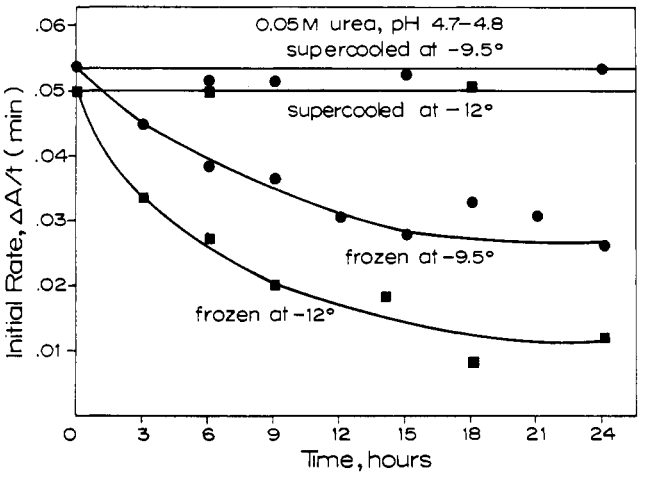

Figure 1. Initial rates of hydrolysis of *N*-acetyltyrosine ethyl ester by enzyme obtained from supercooled and frozen chymotrypsin solutions (0.05 *M* urea) at −12 and −9.5°.

Table I. Denaturation of α-Chymotrypsin in Various 0.05 *M* Solutions at −9.5°

Solute	Time frozen, hr	Activity by TEE[a]	(frozen/supercooled) by normality[b]
None	24	1.00	1.00
KBr	24	0.74 (0.81)[c]	1.04
NaCl	24	0.85 (0.92)	1.03
	48	0.83 (0.97)	0.96
$CaCl_2$	24	0.94	1.06
	48	0.99	1.03
Ethanol	24	0.98	1.00
	96	0.97	
Dioxane	24	0.98	1.00
	48	0.85	0.99
Urea	24	0.50–0.67 (0.55–0.62 after 48 hr at room temperature)	0.38–0.61

Enzyme concentration 0.4–0.6 mg/ml.

[a]TEE denotes *N*-acetyltyrosine ethyl ester analysis (method A). [b]Relative normalities were determined by method B. [c]Parentheses denote analysis after standing 1 hr at room temperature.

With independent trials using different solutions and batches of enzyme, the scatter of activities was such that after 24 hr at −9.5°, analysis by method A gave extremes of 50–60% activity and by method B gave 38–61% activity (Table I). Such variation possibly arises because any kinetic study of this type of "frozen state" reaction requires a completely separate sample for each point of time; this gives rise to potential differences caused by small variations in preparation of each sample, in rate of freezing or thawing, or trace inhibitors or poisons. As it is, calcula-

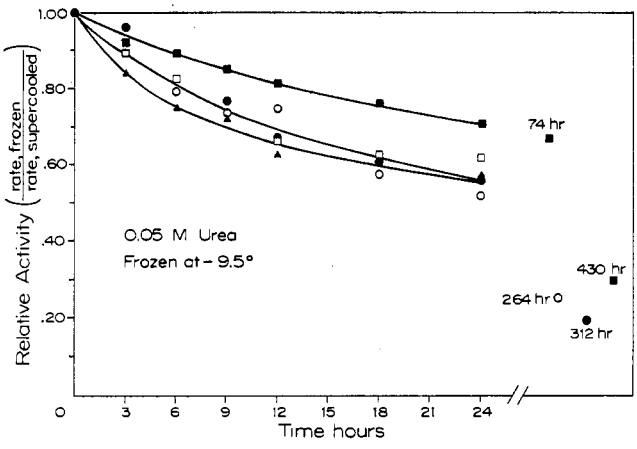

Figure 2. Relative activities of enzyme (toward *N*-acetyltyrosine ethyl ester, method A) in thawed solutions relative to supercooled solutions after being frozen various times in 0.05 *M* urea at −9.5°.

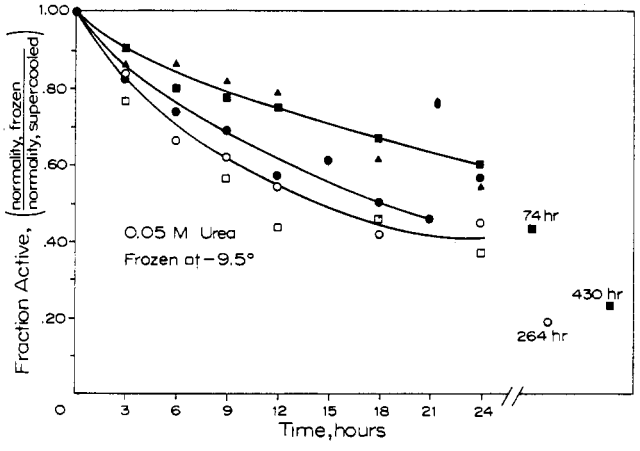

Figure 3. Relative activities of enzyme (by titration method B) in thawed solutions relative to supercooled solutions after being frozen various times in 0.05 *M* urea at −9.5°.

tion of kinetic order or rate constants would be unwarranted and only qualitative comparisons could be made.

Figures 2 and 3 for 0.05 *M* urea solutions show the fraction of activity in runs at −9.5° analyzed by the methods A and B, respectively. The general similarity in loss of activity as determined by the two different methods indicates that some enzyme molecules are completely deactivated at various times, while the remaining enzyme is of normal activity. The deactivation is irreversible: *i.e.*, allowing thawed solutions to stand at room temperature (with or without addition of HCl or $CaCl_2$) did not give increased activity by either method of analysis. As shown in Figures 2 and 3, the denaturation proceeds to about 50% in 24 hr, but is very slow thereafter. This could be caused by about 50% of the enzyme being precipitated or trapped into the ice and therefore remaining unaccessible to urea except for slow thawing and freezing of regions of the ice. Another possibility is that a relatively rapid denaturation by conformational changes occurs, followed by slower autolytic decomposition. In any case, Martin and Frazier (1963) have shown that although α-chymotrypsin in 8 *M* urea at 25° immediately loses all its activity, about 60% is recovered at pH 5.5 by dilution of the urea solution into 0.10 *M* $CaCl_2$. At lower pH's there was a greater recovery of enzyme. At −9.5° the ice-aqueous urea phase relationship would give a urea solution of about 7 *M* in equilibrium in the frozen system (Chadwell and Politi, 1938). Since thawing is a dilution process, and the initial pH in the runs was 4.5–4.8, whatever the process responsible for recovery of *ca.* 60% activity by dilution would occur on thawing of frozen samples (*i.e.*, dilution from 7 *M* back to 0.05 *M*). Frozen samples show an additional slow irreversible denaturation, especially after 25 hr (see Figures 2 and 3), possibly due to autolysis superimposed on the thawing effect.

Effect of Temperature. Figure 1 shows that denaturation in frozen samples is faster and proceeds to a greater extent at −12° than at −9.5°. This reversal of the usual temperature-rate relationship is typical of reactions in frozen systems which are dominated by the concentration effect. More complete data are given in Table II; at −5° little denaturation is attained after 24 hr while the extent of reaction in 24 hr is increased at −10° and further increased at −12°. Minus 12° is most effective with *ca.* 50–75% denaturation in 24 hr. The eutectic point of the urea–water system is −11.4°, at which temperature the urea concentration in the equilibrated liquid phase is 8.08 *M* (Chadwell and Politi, 1938). It is known that denaturation of chymotrypsin in 8 *M* urea is very effective while, say, a 3 *M* concentration has little or no effect. The former concentration corresponds to that which must be present in frozen solutions at *ca.* −12°, while the ice–urea solution phase data also show that the latter concentration would be attained at *ca.* −5°. Samples frozen rapidly below the eutectic temperature of −11.4° would be completely solid in a urea–water system and little denaturation would be expected. However, reaction still occurs slowly at −16°, possibly because some liquid phase is still present. The urea–water phase system will not have the same temperature–concentration relationship nor, more especially, the same eutectic point as the water–urea–chymotrypsin system even if the enzyme is at low concentrations.

Little denaturation shows up in 0.05 *M* samples frozen to −70° and even this may occur as the sample sweeps through the temperature of maximum effect near −12° during the freezing and thawing processes. In general, the temperature effects are consistent with the effect of concentration of urea in the frozen solutions.

Table II. Effect of Temperature on Denaturation of α-Chymotrypsin in Frozen Urea Solutions

	Relative activity = rate frozen/rate supercooled	
Temperature	After 4 hr	After 24 hr
−5°		0.93
−10°	0.82–0.90	0.53–0.70
−12°	0.60–0.75	0.25–0.50
−16°	0.76–0.79	0.60–0.68
−70°	0.90	0.91–0.93

Enzyme concentration 0.42–0.47 mg/ml, pH 4.5–4.8.

Table III. Effect of Added Solutes on Urea Denaturation of α-Chymotrypsin in Frozen Solutions

Sample	Temperature	Time, hr	Activity by TEE	(frozen/supercooled) by normality
0.05 *M* urea	−9.5°	24	0.50–0.67	0.38–61
+0.17 *M* ethanol	−9.5°	24	0.98–1.01	0.89–1.01
+0.85 *M* ethanol	−9.5°	24	1.00–1.01	0.98–1.01
0.05 *M* urea	−12°	24	0.25–0.50	
+0.015 *M* tyrosine	−12°	20	0.29–0.35	
+0.05 *M* $CaCl_2$	−12°	20	0.94–1.03	

Effect of Added Solutes on Urea Denaturation. Ethanol at 0.17 *M* effectively protects chymotrypsin from the effects of 0.05 *M* urea in frozen solutions (Table III). Calcium chloride at 0.05 *M* is also a protective agent. Here the effect is probably a combination of the known decreased rate of denaturation in the presence of Ca^{2+} (Martin and Frazier, 1963) and the well-known inert solute effect in frozen systems. Assuming ideal conditions, the actual concentration of urea in frozen solutions containing various other solutes A, B, . . . will be $[U][0.05\ M\ \text{urea}/(0.05\ M\ \text{urea} + [A] + [B] + \ldots)]$ where $[U]$ is the concentration of urea in equilibrium with ice at a given temperature below freezing (*i.e.*, $[U] = 8\ M$ at 12°) (Pincock, 1969). A solution of 0.05 *M* urea and 0.17 *M* ethanol would give a urea concentration of only $8 \times 0.05/0.22 = 1.8\ M$ in frozen solutions at −12°. A solution containing 0.05 *M* $CaCl_2$ would be as effective as a 0.15 *M* ethanol solute in preventing the buildup of high urea concentration in frozen solutions. On the other hand, solutes initially less than 0.05 *M* would not greatly affect the urea concentration in frozen solutions; *e.g.*, tyrosine at 0.015 *M* is not a protective agent since the urea concentration would still be about 78% of its concentration without any added solute.

CONCLUSIONS

Relative to supercooled solutions the denaturation of α-chymotrypsin in frozen solutions shows rate accelerations, a rate–temperature maximum, and a protective effect of otherwise inert solutes. Although the data could not be made sufficiently precise to allow application of a quantitative treatment, these observed features are common to reactions in frozen solutions which are governed by the concentration effect. At temperatures a few degrees below freezing, and especially with buffers or natural mixtures present, most soluble proteins are not likely to be incorporated in the solid phase. They would be subject to the concentration effect and show the general features of all frozen solution reactions. In addition to these general characteristics, many specific characteristics of enzymes such as reversible conformational changes, denaturation on dilution, thermal denaturation, and autolysis may occur as "secondary effects" in frozen solutions. Since even a single enzyme like chymotrypsin or ribonuclease shows some of these features, the concentration effects during frozen storage of complex enzyme systems would be very difficult to separate out in any detail. However, the freezing of a dilute solution is a way of subjecting an enzyme to a high concentration of solutes yet recovering the sample for easy analysis at the original dilute concentration. Such treatment might be useful for investigating rapid reversible changes brought about by high solute concentrations.

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Rates of Postmortem Metabolism in Frozen Animal Tissues

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Rates of ATP depletion and lactate accumulation in both *pectoralis major* (breast) and *biceps femoris* (thigh) muscles of 18-month-old chickens were determined at several temperatures between +10 and −10°. Rates of these reactions were at least as fast (and in breast muscle significantly faster) at −3° (frozen) as at +10°, +5°, or 0° (unfrozen). The rate of ATP depletion in breast muscle at −3° was significantly slower following immersion in liquid nitrogen than it was following freezing at slower rates to lesser depths. A similar study was performed on *sternomandibularis* (neck) muscles of cutter-grade cows. Rates of ATP depletion and lactate accumulation were significantly faster at −3° (frozen) than at +10° or 0° (unfrozen).

As postmortem muscle goes through rigor mortis, it undergoes a series of complex biochemical and physical changes. The time changes of several chemical and physical properties of beef *sternomandibularis* muscle and horse *longissimus dorsi* muscle during rigor mortis at 37° have been reported, respectively, by Newbold (1966) and Lawrie (1953). These relationships are very similar and feature the following main points: almost immediately after slaughter, a decline is observed in pH and creatine phosphate content; adenosine triphosphate (ATP) remains virtually constant until the creatine phosphate content has been reduced substantially; and the decrease in muscle extensibility is not appreciable until ATP has

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