CONFIGURATIONAL TRANSITION IN LYSOZYME

JOHN G. FOSS

Department of Chemistry, University of Oregon, Eugene, Oreg. (U.S.A.)

(Received January 16th, 1960)

SUMMARY

Experimental techniques have been developed for conveniently measuring the temperature dependence of rotation of proteins using relatively small quantities of material. Results of rotation-temperature studies on lysozyme in aqueous urea solutions are presented and compared to earlier results with ribonuclease. Both the ribonuclease and the lysozyme undergo an inverted configurational transition in concentrated urea solutions; *i.e.*, some protein goes from a "denatured" to a "native" state when the temperature is increased. The reversal of the transition was also studied and found to be first order in lysozyme and to have a large activation energy.

INTRODUCTION

During recent years a great deal of work has been done in studying the physicochemical properties of synthetic polypeptides[1]. The studies were carried out not only because these newly available compounds are intrinsically interesting but also because it was hoped they might be satisfactory model compounds for the naturally occurring proteins. In many ways the latter hope has been borne out.

We have recently published a study of ribonuclease[2] which was undertaken to see if, in a sense, the proteins could be used as model compounds for synthetic polypeptides. In particular the work was initiated to see if it was possible to observe an effect DOTY AND YANG found[3] for PBGA in a mixed solvent and termed by them an "inverted transition". They found that on raising the temperature of the PBGA it went from a less ordered to a more ordered state. In a protein such an inverted transition would correspond to renaturing a partially denatured protein by elevating its temperature. As is well-known this is certainly not the normal behavior of proteins.

Denaturation is normally defined in operational terms and for convenience we chose to define it in terms of the same easily measurable physical characteristic used by DOTY AND YANG viz., the optical activity[4-6]. Using this criterion for denaturation we demonstrated the existence of inverted transitions for ribonuclease in concentrated urea solutions. Admittedly, ribonuclease with its twenty odd amino acids and four disulfide cross links is a very complex "model compound" for PBGA though it does have the advantage of having a very well defined molecular weight. On the other hand it is a good "model protein".

Abbreviation: PBGA, polybenzylglutamate.

The optical rotation is a measure of the average environment of the optically active centers of the protein. Thus any changes in structure of the polypeptide "backbone" of the protein (where most of the asymmetric centers are located) will result in a change in the optical activity. For this reason the more descriptive term "configurational transition" will be used to characterize the type of denaturation to be discussed in this paper. Such a transition may be induced by raising the protein temperature, adding a strong hydrogen bond former to the protein solution or by a combination of these methods.

A number of questions were raised by the study on ribonuclease, but before returning to them we decided to initiate the present experiments on lysozyme. There were several reasons for doing this, but the most important was the desire to develop procedures for working with smaller quantities of material since we were using as much as 300 mg for a single experiment with ribonuclease. In addition we wished to see what similarities there are between the behavior of ribonuclease and lysozyme.

In this paper only those effects on lysozyme which result from the presence of a strong hydrogen bond former (urea) will be considered. Consequently, the experiments were all carried out at essentially the same pH (near 6) and at moderately high ionic strength (0.06 to 0.1).

EXPERIMENTAL

Armour crystalline egg white lysozyme Lot D-638040 was used for all of the experiments. This lot contained 9.0 % moisture as determined by drying at 100°. The solutions were all made up in 0.1 *M* potassium chloride. This solution was either added directly to the protein or else to the required amount of solid urea. None of the solutions were buffered. The urea was Baker and Adamson reagent grade recrystallized once from ethanol.

Rotations were measured with a precision Rudolph polarimeter with a rocking polarizer. Unless indicated otherwise all of the measurements were made at the strong mercury 365 mμ line. In the earlier experiments with ribonuclease all measurements of optical activity were made at 546 mμ rather than the traditional sodium D line since proteins have a higher specific rotation at this wavelength. In order to reduce the sample size the logical step was to go to still shorter wavelengths. In principle it should be possible with proteins to obtain increasingly great specific rotations to about 200 mμ, but in practice it is not feasible to work below 300 mμ because of the strong absorbance of the protein tyrosine, phenylalanine and tryptophan groups. In addition since light scattering increases markedly with decreasing wavelength, scattering losses can be serious. At present the 365-mμ line seems to be a satisfactory compromise.

The first measurements of the temperature dependence of rotation were made in a commercial (Rudolph) 1 dm glass polarimeter tube with a 3 mm bore. Light fluctuations discussed earlier are not as serious in the 3 mm tube as in the 8.5 mm tube used previously. Normally 1.5 ml of 1 % protein solution was prepared, so 15 mg of material was used for each experiment. It was found necessary to observe a number of precautions in studying the temperature dependence of rotation. Quartz windows should not be used because they exhibit a large temperature dependence of rotation with some hysteresis. Even with glass windows large changes (*e.g.*, 0.2°) were occasio-

nally observed on heating polarimeter tubes to 80°. These rotations were probably the result of stresses set up in the glass because the differential expansion of the glass and metal in the tubes was not being taken up by the rubber gaskets. Several experiments were carried out in which the tubes were cycled through temperature changes between room temperature and 80°, and it was found that when large changes did occur it was only on the first cycle. For this reason these polarimeter tubes were routinely heated and cooled before making measurements at varying temperatures.

Because of these difficulties a new type of polarimeter tube was devised which proved much more satisfactory for this work. It was made from a rod of polymethylmethacrylate 2.99 cm in diameter and 10 cm long. A 3 mm wide slot was milled the length of the rod to a depth of 1.64 cm with a round bottomed tool. At first glass windows were cemented to the ends, but it proved more satisfactory to simply stick them on with silicone grease since birefringence effects were observed in the cemented windows when heated. Two tapered pins were used at each end of the tube to keep the windows from sliding down. The top surface of the tube was milled flat to permit the use of a flat piece of plastic for a cover. Solutions in tubes of this type respond very sluggishly to temperature changes in the constant temperature compartment of the polarimeter so the solution temperature was measured directly with a Veco 41A11 glass covered thermistor in a bridge circuit. Apart from the slow response to temperature changes these tubes have proven to be superior in almost all ways to the commercial jacketed capillary tubes. They are easy to fabricate, fill, clean and assemble. Normally 1.5 ml of solution is used in a 1 dm tube with a 3 mm slot, but this can be reduced to approx. 1.0 ml. Shortly before completing this work a polarimeter tube of this type was made from a solid rod of titanium. It has a more rapid temperature response and can be used at higher temperatures.

While using these tubes it was found that the simplest and most satisfactory way to clarify protein solutions was to filter them through a 0.5 μ Millipore filter with a syringe adaptor.

Viscosity measurements were made in Cannon-Ubbelohde semi-micro dilution viscometers (Size 25) having flow times of about 450 sec with water at 25°. The viscometers were calibrated from 25 to 60° with water. Since dust particles were troublesome the solutions were centrifuged, but the author would recommend the use of Millipore filters instead.

One of the most serious problems in making viscosity measurements was with frothing. By the judicious use of reduced pressure on the third tube of the Ubbelohde viscometer as well as on the capillary tube, it was possible to keep bubble formation to a minimum. In addition a thin gold wire was inserted in the viscometer in such a way that the end was just above the upper reference line. Many bubbles could be broken by raising and lowering them past this wire.

A Spinco Model E ultracentrifuge was used for several measurements discussed below. A Beckman Model G pH meter was used for all pH measurements.

RESULTS

Fig. 1 shows the rotation*-temperature curves for lysozyme in 0.1 *M* potassium chloride and in 8 and 10 *M* urea solutions. The latter solutions were prepared by adding

* In all cases in this paper when the word "rotation" is used it means levorotation.

0.1 *M* potassium chloride to the required amount of solid urea and consequently have a somewhat lower ionic strength. The urea solution was then added to a sample of protein.

These curves were obtained by slowly raising the temperature and observing the rotations. Several hours were required to complete each experiment in the figure and under these conditions the rotations were only partially reversible (except in the case of 6 *M* urea to be discussed later). Two experiments in the absence of urea are shown in the drawing. In the first a freshly prepared lysozyme solution was heated from 25 to 67° and no appreciable change was observed in the rotation. The observed value at 25° is in excellent agreement with that recently reported by JIRGENSONS[7]. It was not possible to make measurements above 67° as the solutions became turbid. This turbidity is not caused by the precipitation of the lysozyme but by the precipitation of an impurity. That it is an impurity is shown by the fact that if a solution of lysozyme is heated and then filtered it does not become turbid on reheating to the same temperature. In addition the sedimentation pattern of the unheated solution shows a distinct (but very small) fast-moving peak. A one percent solution of lysozyme made up in 0.004 *M* mercuric chloride lacks the small peak in the sedimentation pattern and is slightly turbid when first prepared. Thus it appears that the impurity is precipitated by mercuric ions.

From an examination of the experiments in urea solutions (to be discussed below) it appeared that the thermal transition for lysozyme in 0.1 *M* potassium chloride should be half completed at 78°. After removing the impurity it was possible to make the required measurements in the titanium polarimeter tube. The results are shown in the second 0 *M* curve in Fig. 1. Measurements were not made beyond 82° as it was not possible to go to higher temperatures at the time the experiment was performed. However, the results clearly show that the transition is half-completed near 78° as predicted. With a modification of the experimental arrangement it became possible to go to higher temperatures, but it was found that the rotation only slowly increased on increasing the temperature. In order to carry out measurements through the entire transition it will be necessary to use a modified experimental set-up as evaporation becomes a serious problem when the solutions are heated for long periods at these temperatures.

To remove the impurity prior to these measurements the following procedure was used. 1 % lysozyme solutions in 0.1 *M* potassium chloride were heated 5 min at 90°. The small amount of cooked-egg-white-like material that formed was spun down in a clinical centrifuge. Since the exact lysozyme concentration was no longer known it was estimated by assuming a specific rotation of 201°; *i.e.*, it was assumed the protein remaining in solution was in its native state. It was possible to calculate the specific rotation of the protein remaining in solution by estimating the weight of the precipitate. The calculated specific rotation at room temperature was closer to 220 than to 201° suggesting some irreversible change in the heated protien. For this reason attempts are now being made to purify the lysozyme by a less drastic procedure.

Two additional experiments were performed in 4 and 6 *M* urea but are not shown in Fig. 1. In the 4 *M* solution it was not possible to complete the measurements on the transition since the impurity began to precipitate. However, it was possible to quite accurately estimate the temperature for the middle of the transition as 62.5°.

The 6 *M* experiment is especially interesting in that the rotation was completely reversible although the solution was heated from 13 to 75° during a 6-h interval. The transition was half complete at 56.2°. In several separate experiments rotation-temperature curves were obtained for lysozyme in 8 *M* urea over a period of several hours. At the completion of the experiments the solutions were allowed to stand overnight at room temperature. In all cases the final rotations were close to 230°. However, it is possible to obtain complete reversibility by heating these solutions for short periods of time. For example, on heating such a solution for 5 min at 80° and then suddenly cooling to 25° the rotation will return to its initial value in less than 15 min. (During this 5-min interval the rotations do increase to the values expected but it is difficult to obtain accurate measurements.) Unlike the experiments in 10 *M* urea the measurements in 8 *M* solutions were quite reproducible. Several experiments gave results that were identical within experimental error.

The reason for this irreversibility is not clear, though the possibility of oxidation by air during the heating appears to have been ruled out by the following experiments. Two solutions of lysozyme in 8 *M* urea were prepared that differed only in that one was saturated with nitrogen and the other with oxygen. Both were heated to 80° for 1 h and then cooled to room temperature. The levorotation of both solutions had increased about 60°.

Experiments performed in 10 *M* urea solutions give a variety of curves displaced vertically from one another; *i.e.*, the temperatures of the minima and maxima are essentially unchanged. For example Fig. 2 shows the results obtained in another experiment under conditions very similar to those of Fig. 1. Initially the levorotation was 238° at room temperature. When the temperature was raised the levorotation increased to a maximum of 284.5° and then started to decrease (total time elapsed between minimum and maximum temperature readings was 2 h). The solution was then quickly cooled to 25° and left overnight. The following day the upper curve was obtained when the sample was reheated. This solution was then cooled and diluted with 0.1 *M* potassium chloride to bring the urea concentration to 5 *M*. In less than 5 min the levorotation was 223°. Thus the lysozyme in the 5 *M* solution had apparently largely reverted to a native-like state as judged by the marked drop in levorotation and the large change in rotation on reheating.

Fig. 1 also includes the results of an experiment in which the viscosity of a lysozyme solution in 8 *M* urea was measured at various temperatures. The concentration of the protein was about 3 % and 1 ml of solution was used. The purpose of the experiment was to learn how close a parallelism existed between changes in viscosity and rotation. Since the rotation and viscosity curves have the same sigmoid shape the viscosity data were fit to the rotation data by equating the distance between the minima and maxima of the curves. As can be seen from the figure the correlation between viscosity and rotation changes in the transition region is striking.

It was found previously that when 7 *M* urea solutions of ribonuclease at 50° were suddenly cooled the levorotation increased rapidly before slowly returning to smaller values. A similar effect was looked for with lysozyme and first observed in the 10 *M* urea solution. If such a solution is rapidly cooled from 52° to 25° the rotation observed initially is on an extension of the negative slope of the temperature-rotation curve as indicated by a dotted line in Fig. 1. The rotation will not return to its original value on cooling if it remains at elevated temperatures for a long period. However, if

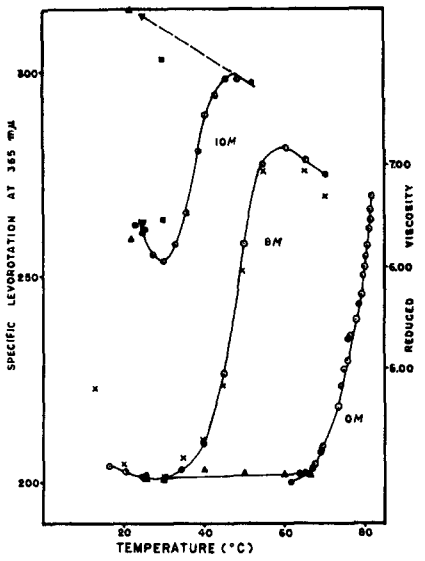


Fig. 1. Temperature dependence of levorotation and viscosity of lysozyme in 0, 8 and 10 *M* urea. (All solutions approximately 0.1 *M* in potassium chloride.) △ and ⊙ represent two separate experiments in 0 *M* urea. The crosses represent viscosity data in 8 *M* urea. The symbols ⊡, ▽ and △ near the 10 *M* curve represent reversal data at 30, 25 and 22° respectively. (See text and Fig. 3.)

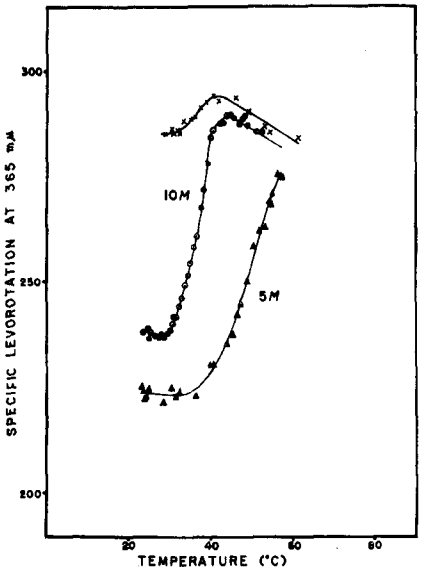
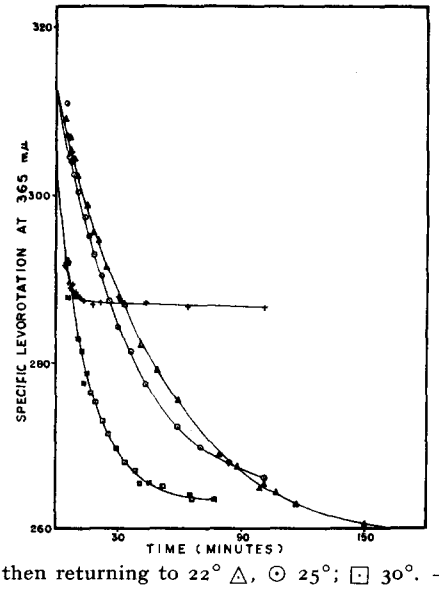


Fig. 2. Temperature dependence of levorotation of lysozyme in 10 and 5 *M* urea (plus approximately 0.1 *M* potassium chloride). ⊙, Freshly prepared solution of lysozyme in 10 *M* urea. ×, Same solution after standing overnight at room temperature. △, Same solution after diluting to 5 *M* in urea.



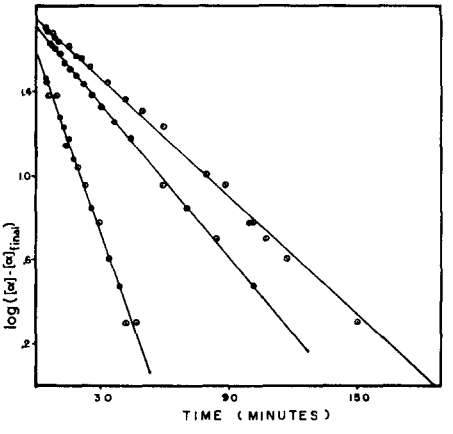


Fig. 4. First order plot of reversal data of Fig. 3.

Fig. 3. Time dependence of levorotation of lysozyme in 10 *M* urea after heating for 5 min and then returning to 22° △, ⊙ 25°; ⊡ 30°. + indicates a reversal at 25° but in the presence of mercuric chloride.

a solution is kept at 80° for only 5 min and then rapidly returned to 25° the rotations are largely reversed.

Some experiments of this kind are shown in Fig. 3. Three of the curves show the results of reversals to three different temperatures viz., 22, 25 and 30°. Unfortunately, it is difficult to extend the temperature much beyond this since the 10 *M* urea begins to precipitate below 22° and the rate is too fast to measure accurately above 30°. (It might be possible to make such measurements at lower temperatures by using formamide instead of urea as the hydrogen bond breaker.) These experiments were performed by heating the samples in closed test tubes at 80° for 5 min, cooling for 1 min in a water bath at the desired final temperature and then rapidly transferring to the plastic polarimeter tube. The latter was kept in the constant temperature compartment for about 30 min before adding the cooled protein solution. Since a thermistor was not available at the time these experiments were performed there may be a small error in the final temperature because of temperature changes during the transfer, etc. In Fig. 4 these results are plotted to show the goodness of a first order fit. (This fit was made by adjusting the rotations at infinite time to give the best straight lines. Though this may seem arbitrary, the resulting final rotations calculated in this way are in very good agreement with the values that would be estimated from Fig. 3). From the intercept the initial rotation can be calculated and these values are shown in Fig. 1. Two of the values are on the extension of the upper slope. Fig. 3 also includes an experiment in which mercuric chloride was present at a concentration of 0.004 *M*. The presence of the mercuric ions apparently partially blocks the reversal process.

DISCUSSION

General interpretations

The results of Fig. 1 are qualitatively the same as those obtained for ribonuclease[2] and it is proposed to interpret them in the same manner. These interpretations can be summarized as follows: (a) As the temperatures of the lysozyme solutions are increased they all show a marked increase in rotation. This is caused by a configurational transition in the protein. (b) The 10 *M* urea solution has the smallest amount of native protein present at the beginning of the transition and therefore exhibits the smallest change in rotation. (c) At temperatures above the maxima in the rotation-temperature curves the slopes are parallel since only denatured protein is present. The slope gives the intrinsic temperature dependence of rotation of unfolded protein. Rotations in solutions having different refractive indices are not directly comparable[8] and for this reason the upper slopes do not lie on a common line. For ribonuclease there was clear-cut evidence for this interpretation as the upper slopes did fall on a common line after refractive index corrections were applied. The data presented in Fig. 1 cannot be interpreted so easily, and we must argue in part by analogy with ribonuclease. No refractive index data are available for concentrated urea solutions at 365 mμ though it should be possible to make the necessary measurements in this laboratory in the near future. But even with the corrections it is clear that the upper slopes cannot all coincide. For example, if it had been measured, the upper slope in the 0 *M* solution could not coincide with that of the 8 *M* solution even after being corrected. The reason for this is that the corrections will reduce the rota-

tions for the 8 *M* but not for the 0 *M* solution. However, judging from the corrections at 589, the upper slopes for the 8 and 10 *M* solutions will probably coincide after the refractive index corrections have been made. (It has been shown that these corrections are not strongly dependent on wavelength[8].) (d) Below the temperatures of the minima in the rotation-temperature curves an inverted transition is occurring; *i.e.*, some protein is being denatured as the temperature is decreased. (e) At temperature below the minima in Fig. 1 the slope is greater for the 10 *M* solutions for two reasons. First in 10 *M* urea more of the unfolded lysozyme is present at low temperatures than in the 8 *M* solution as judged from the larger rotation. Since the unfolded material has a larger intrinsic temperature dependence of rotation, a steeper slope is observed for the 10 *M* solution. Secondly, the inverted transition will lead to a greater decrease in rotation as the temperature is increased.

The arguments for these interpretations are given in greater detail in the previous paper on ribonuclease.

On comparing the data of Fig. 1 with the comparable data for ribonuclease it is apparent that lysozyme is considerably more stable under the conditions of the experiments. For example, judging from the change in rotation no configurational change has occurred in lysozyme on heating at 70°. In ribonuclease, however, the protein has gone through the entire transition by 72° *i.e.*, the maximum in the rotation-temperature curve is observed at this temperature. In 8 *M* urea ribonuclease is completely unfolded even at 5° while lysozyme only begins to unfold at 30°.

Thermodynamics

Although the rotation-temperature data are not completely reversible, it is possible to make use of the VAN 'T HOFF relationship to estimate the change in enthalpy on unfolding[11]. However, as mentioned in the RESULTS the rotation-temperature data for lysozyme in the absence of urea are changing markedly with time. For this reason and because it was not possible to satisfactorily complete the transition, the calculations have been made for the 8 *M* urea solution only. Plotting the logarithm of the equilibrium constant *versus* the reciprocal of the absolute temperature gives a reasonably straight line with a slope corresponding to 69 kcal. If a transition temperature is defined as that temperature at which the equilibrium constant is unity, a value of 47° is obtained and the entropy of unfolding at this temperature is 215 eu. It would be desirable to learn what the change in enthalpy is in the absence of urea. This would permit a comparison of the relative numbers of hydrogen bonds broken during the transition for the ribonuclease and lysozyme molecules assuming a negligible difference in side chain effects.

There has been only one comparable measurement reported to this writer's knowledge. HAMAGUCHI[9] has measured the temperature dependence of viscosity of lysozyme in 8 *M* urea and used a two state model to determine the enthalpy change. He reports a value of approx. 5 kcal and argues that this unusually small value implies a small structural change in the lysozyme on unfolding. However, it appears that an error was made in his calculations since HAMAGUCHI's own data leads to a value of 52 kcal and a transition temperature of 41°. Whether this difference between HAMAGUCHI's data and ours reflects a real difference in the lysozyme samples used or in the experimental procedure is not clear at present.

Recently a number of papers have been published discussing the statistical

mechanics of the phase transition occurring in polypeptides[10–15]. In one of these PELLER has shown that

$$T_t^\circ - T_t \approx \frac{R(T_0)^2}{\Delta h} 2KC$$

where T_t° is the transition temperature in the absence of denaturant, T_t is the transition when the denaturant is at concentration C. K is the average equilibrium constant for denaturant binding to the sites exposed after the configurational transition and Δh is the heat of binding for the same process. Since Δh is known to be small (approx. — 2 kcal) the temperature dependence of K may be neglected as a first approximation. Using reasonable values PELLER has estimated a value of — 4.44 for the coefficient of C. The data for lysozyme and ribonuclease are in remarkably good agreement with this relationship as can be seen from Fig. 5. If the data for lysozyme are extrapolated to 0 M the transition temperature is found to be 78°. This extrapolation was made before any experiments had been successfully completed in the absence of urea and the prediction provided an impetus for completing such an experiment. As mentioned in the RESULTS it was not possible to very accurately measure the transition temperature but it is probably within one degree of the predicted value. Thus the transition temperature for lysozyme varies linearly with the urea concentration over the entire experimental range of 0 to 10 M urea. This agreement is additional evidence for the applicability of the very simple model used for the configurational transitions of proteins. The slope for the lysozyme data is −3.3 and that for the ribonuclease is −4.5. These are of the expected order of magnitude.

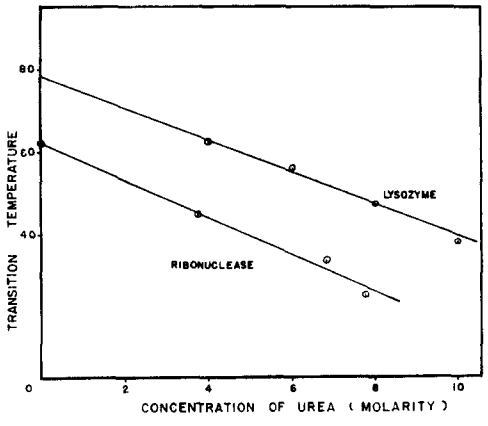


Fig. 5. Dependence of transition temperature of lysozyme and ribonuclease on concentration of urea.

However, if we assume common values for K and Δh for all proteins the slope for the lysozyme should be somewhat greater since its transition temperature in the absence of urea is greater. This suggests that such an assumption is incorrect, which is not surprising.

PELLER has also pointed out the interesting similarity between this equation for the transition point depression and the usual expression for the freezing point depression of a solvent by the addition of solute. The folded protein would correspond to microcrystals of pure solvent in the solid phase and the unfolded protein to protein

in the liquid phase. This would agree with KAUZMANN's suggestion[16] that protein configurational transitions are similar to the melting of ordinary crystals but with a relatively broad "melting point", because the crystal size is so small.

Fig. 5 also shows quite clearly that the 5 *M* solution of Fig. 2 is quite different in its behavior from that expected for native lysozyme. While the $\alpha(T)$ curve was not carried to completion a reasonable estimate of the midpoint of the transition would be 50°, but Fig. 5 shows that a value of 59° would be expected. One simple interpretation of this behavior is that the refolding which takes place on removing the urea does not occur in the most efficient manner; *i.e.*, the manner which would put it into its state of lowest free energy. An interesting corollary of this is that if one were to very slowly remove the urea the final state might more closely approximate the native protein. Similarly, cooling a protein slowly might help to return it to a more native-like state since this would increase the rate of attainment of equilibrium unless of course irreversible effects take place during the time it is at the elevated temperature.

Kinetics

The rapid reversal experiments described are quite interesting and in part qualitatively different from earlier reports on the reversal of protein denaturation. The temporary entrapment of the protein in the high temperature, unfolded form is similar to a supercooling effect in an ordinary phase transition. We have attempted to evaluate the activation for this reversal process but the data are not accurate enough to give reliable values. It is clearly very large (probably greater than 100 kcal) and consequently any small error in the sample temperature on cooling will give rise to marked changes in the velocity of refolding. (As mentioned earlier, there may have been small errors in the temperatures used for the activation energy calculations. These can be eliminated by the use of the titanium polarimeter tube and the direct measurement of temperature with a thermistor probe.)

Only one experiment is shown in Fig. 3 in which mercuric ions were used to partially block the reversal. Such experiments might indicate what groups participate in the refolding. For example it is thought that mercuric ions may bind weakly to histidine side chains in papaya lysozyme. It may also be possible to more directly get at this question of why agents such as mercuric ions block the reversal by following the reactions with difference spectra measurements. Some preliminary measurements (but in the absence of blocking agents) have been made of the difference spectrum between an unheated sample of lysozyme in 10 *M* urea and a sample temporarily trapped in the unfolded state. Two distinct peaks located near 292 and 237 mμ have been observed which decrease with time. These promising experiments are being continued.

ACKNOWLEDGEMENTS

The author wishes to thank the U.S. Public Health Service for financial support, J. A. SCHELLMAN for many helpful discussions, R. W. GOODRICH for the machine work on the polarimeter tubes and D. M. MORTIMORE of Oremet for a gift of titanium rod.

REFERENCES

[1] C. H. BAMFORD, A. ELLIOTT AND W. E. HANBY, *Synthetic Polypeptides*, Academic Press, New York, 1956.
[2] J. G. FOSS AND J. A. SCHELLMAN, *J. Phys. Chem.*, 63 (1959) 2007.
[3] P. DOTY AND J. T. YANG, *J. Am. Chem. Soc.*, 78 (1956) 498.
[4] W. KAUZMANN, *Ann. Rev. Phys. Chem.*, Vol. 8, Palo Alto, 1958.
[5] W. KAUZMANN AND H. EYRING, *J. Chem. Phys.*, 9 (1941) 41.
[6] C. SCHELLMAN AND J. A. SCHELLMAN, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 30 (1958) 463.
[7] B. JIRGENSONS, *Arch. Biochem. Biophys.*, 74 (1958) 70.
[8] J. A. SCHELLMAN, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 30 (1958) 439.
[9] K. HAMAGUCHI, *J. Biochem. (Tokyo)*, 45 (1958) 79.
[10] J. A. SCHELLMAN, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 29 (1955) 230.
[11] J. A. SCHELLMAN, *J. Phys. Chem.*, 62 (1958) 1485.
[12] L. PELLER, *J. Phys. Chem.*, 63 (1959) 1194, 1199.
[13] B. H. ZIMM AND J. K. BRAGG, *J. Chem. Phys.*, 28 (1958) 1246.
[14] J. H. GIBBS AND E. A. DIMARZIO, *J. Chem. Phys.*, 28 (1948) 1247.
[15] T. L. HILL, *J. Chem. Phys.*, 30 (1959) 383.
[16] W. KAUZMANN, in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, The Johns Hopkins Press, 1954.

Biochim. Biophys. Acta, 43 (1960) 300–310

MECHANISM OF INHIBITION OF D-AMINO ACID OXIDASE

IV. INHIBITORY ACTION OF CHLORPROMAZINE

KUNIO YAGI, TAKAYUKI OZAWA AND TOSHIHARU NAGATSU

Dept. of Biochemistry, School of Medicine, Nagoya University, Nagoya (Japan)

(Received February 15th, 1960)

SUMMARY

1. The interaction of chlorpromazine and FAD was studied in aqueous solution and in a reaction mixture containing D-amino acid oxidase. The formation of a complex between chlorpromazine and FAD and the competition of these compounds for the apo-enzyme were demonstrated.

2. The formation of complexes of chlorpromazine and flavins was demonstrated by means of spectrophotometry and fluorimetry. The dissociation constant of the complex of chlorpromazine with FAD at pH 7.0 and 20°, measured by fluorimetry, appeared to be $1.0 \cdot 10^{-3}$ *M*. The same dissociation constant was found for the chlorpromazine complexes with riboflavin, FMN or riboflavin 5′-monosulfate.

3. The competition of chlorpromazine with FAD for the apo-enzyme was demonstrated by means of kinetic analysis. The dissociation constant of the complex of chlorpromazine with the apo-enzyme at pH 8.3 was calculated to be $2.3 \cdot 10^{-5}$ *M*.

4. The binding site of the apo-enzyme with chlorpromazine was determined by using riboflavin 5′-monosulfate and adenosine 5′-monosulfate as indicators. The results showed that chlorpromazine competes with adenosine 5′-monosulfate for the apo-enzyme, which means that chlorpromazine binds at the same site as the AMP moiety of FAD.

Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin monophosphate; AMP, adenosine monophosphate; FMS, riboflavin 5′-monosulfate; AMS, adenosine 5′-monosulfate.