Heat of Transition of Ribonuclease A[1]

K. Beck, S. J. Gill, and M. Downing

Contribution from the Department of Chemistry, University of Colorado, Boulder, Colorado. *Received September 24, 1964*

Calorimetric measurements have been made on the thermal transition of ribonuclease A in a buffered solution of pH 2.8. Solutions with concentrations of 1.5 and 3% by weight were examined between 20 and 60°. A heat of transition of 70 ± 1 kcal./mole was found for both of these concentrations. The same value was reproduced on a repeat measurement of a solution that had been subjected to a thermal transition, thereby confirming the reversibility of the effect that causes the transition enthalpy. The same value was obtained upon a twofold change in the rate of temperature elevation, from approximately 1.5°/hr. to 0.8°/hr. The thermally treated and untreated ribonuclease solutions gave similar disk electrophoresis patterns. The heat capacity change upon transition was found to be 0.66 kcal./mole-deg. A measurement of the heat required to promote a certain portion of the transition provides another route for describing the fraction of transition as a function of temperature. From these results one concludes unequivocally that the transition consists of more than one step.

Introduction

A number of studies have been made on the reversible thermal transition of ribonuclease[2-7] in order to understand the mechanism of the transition. Scheraga has also reviewed much of this work in terms of a model for the configuration of ribonuclease.[8] One feature of these studies has been to interpret the transition in terms of thermodynamic parameters, for example, an equilibrium constant. In order to calculate an equilibrium constant, it is necessary to assume a particular reaction mechanism and further to assume that the property studied during the course of transition can be translated into the terms of concentrations in the equilibrium constant expression. Under these circumstances, it has been natural to choose mechanisms which are characterized by simplicity, in particular, the single step all-or-none reaction or a two-step reaction process.[6,7] From the temperature dependence on the equilibrium constant the heat of the assumed reaction can be determined.

A direct calorimetric determination of the heat of the transition would provide the necessary information to differentiate between some of the simpler mechanisms of the transition.[6,7,9] Apart from a test of possible reaction mechanisms, the enthalpy of the transition is an important parameter describing the energetics of the ribonuclease molecule. In the work reported in this paper, we have restricted the experimental conditions to acid solutions of pH 2.8 so that the calorimetric values might be compared to the work of several investigators.[4,6,7]

Experimental

Materials. Ribonuclease A was obtained from Worthington Biochemicals, Inc., as the lyophilized phosphate-free form. Gel electrophoresis of the material dissolved in water showed the sample consisted of better than 99.9% of one component. Solutions were prepared from material which had been dried to constant weight at 105°. The solutions were made to conform as closely as possible to the pH and ionic strength conditions of Holcomb and Van Holde[6] by use of 0.01 *M* potassium acid phthalate and 0.15 *M* KCl with the pH adjusted to 2.80 with HCl. A preliminary viscometric study of the test solution was made, and the results conformed to those obtained by Holcomb and Van Holde.[6]

Calorimetric Measurements. The calorimeter used in this study is a high-sensitivity differential heat capacity instrument.[10] The heart of the apparatus consists of two copper disks, 2.5 in. in diameter, separated by a thermopile of 140 junctions.[11] Heaters of constantan wire are located in concentric grooves over one external face of each copper disk. Heaters are also located on the back faces of a ring turned from the copper disk for calibration purposes. Both disks are made as symmetrical as possible. The solution to be measured is placed in a pill-box shaped container, made of tantalum with a closure disk 0.015 in. thick to act as a diaphragm for volume changes of the solution. The container is sealed vacuum tight by means of a Viton o-ring. Two such cells were made to hold approximately 12 ml. In operation one cell was filled with ribonuclease solution and the other with buffer solution.

The copper disk and solution cell unit is suspended within a closed copper cylinder (5-in. diameter and 7-in. length) that is a thermocouple-controlled adiabatic shield. The copper adiabatic shield is in turn concentrically nested within a heavy walled aluminum cylinder with end plates and o-rings to provide vacuum-tight surroundings. The aluminum cylinder and end plates also act as a thermocouple-controlled adiabatic

(1) Based in part on a dissertation submitted by K. B. to the Graduate School faculty, University of Colorado, in partial fulfillment of the requirements for the Ph.D. degree, 1964.

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(10) A detailed description of the apparatus is given in the Ph.D. Thesis of K. B., University of Colorado, 1964, and has been submitted for publication in *Rev. Sci. Instr.*

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shield. The aluminum adiabatic shield is located within an air thermostat which is controlled to follow the temperature of the aluminum shield.

The operation of the calorimeter consists of electrically heating the face plates of the central copper disks to provide a given rate of temperature increase. The electrical heating of each disk is so adjusted with disks well below the polymer transition temperature as to minimize the temperature difference, as indicated by the central thermopile voltage. The thermopile voltage is amplified by a Beckman Model 10 amplifier and recorded by a Brown recorder. Any change in the heat capacity of the units on either side of the central thermopile will cause a temperature difference between the disks to occur with a consequent deflection of the e.m.f. trace from the original baseline. This deflection is also proportional to the increase or decrease in the rate of heat transfer between the two disks. Thus the area under the deflected trace is proportional to the additional amount of heat required during a transition reaction. In order to find the proportionality constant which translates this area into units of heat, it is necessary to calibrate the system with a known heating process.

The calibration has been performed in two ways. The first way is to provide an additional small amount of constant electrical heating to one disk or the other by means of a constant measureable current into one of the back ring heaters on the disk. When a steady-state deflection is achieved, the proportionality constant can be readily determined. From calibrations of this sort it has been found that the calibration constant is the same for both sides and that it remains constant over the range of temperature in which the calorimeter was used.

The second method of calibration is actually a check on the first method. It consists of placing a given amount of an encapsulated solid into one side of a solution cell. In this way one simulates the transition effect of the polymer by the phase transition of a known solid. Diphenyl ether was particularly appropriate for the ribonuclease studies since it has a melting temperature of 26.8° and the purified material has been carefully studied by Furukawa of the National Bureau of Standards.[12] An amount of recrystallized reagent, 25 mg. of diphenyl ether, was sealed within a short section of melting point tubing and placed within the solution calorimeter cell filled with buffer. The calorimeter was then operated through the transition region of the diphenyl ether, and from the area under the deflection curve along with the electrical calibration constant a value of 100.3 joules/g. was determined for the heat of transition. This compares favorably with the value given by Furukawa of 101.15 joules/g.[12]

The temperature of the calorimeter was read periodically by means of a standard thermocouple and also checked by means of the air bath temperature with a standard thermometer. The temperature was therefore known to approximately ±0.1°.

Results and Discussion

A typical trace of the e.m.f. of the central thermopile *vs.* temperature or time is shown in Figure 1. As can be seen, displacement of the baseline appears after the transition has been completed. This displacement is interpreted as the difference in the heat capacity between the denatured and natural material. We have assumed that the baseline during the course of the transition could be described by a straight line between the point prior to the transition to the point after the transition. From the area under the curve the heat of the transition was evaluated.

The first set of runs are indicated by I, II, and III in Table I. These runs were made on the same solution. The first run (I) was carried out to a temperature of 60° at a rate of heating that gave about a 1.5° temperature rise per hour. The calorimeter was then cooled overnight to a temperature of 20°, and the run was repeated at the same rate of heating but to a final temperature of 63° (run II). This slightly higher temperature of run II was used in order to check the completion of the transition at the risk of permanently denaturing a part of the sample. The transition curves for I and II were virtually superimposable, thus showing the thermal phenomena being measured was completely reversible. Run III was made at a much slower rate of temperature change per hour in order to test for a lack of equilibration at the previous rates. The measured amount of heat in run III was slightly lower than the amount recorded for runs I and II, and is considered to be due to a slight amount of permanent denaturation at the end of run II and not to any effect of the slower rate of temperature change.

Table I. Enthalpy Measurements on Ribonuclease A Transition, pH 2.8[a]

	Run 1	Run 2	Run 3	Run 4
Weight per cent ribonuclease	2.690	2.690	2.690	1.385
Heating rate, deg./hr.	1.5	1.5	0.8	1.5
Enthalpy, kcal./mole	+70.1	+69.1	+64.1	+70.8
Temperature range, °C.	20–60	20–63	20–62	20–62
Transition midpoint, °C.	43.2	43.2	43.2	43.1

[a] The same solution was used in runs 1, 2, and 3.

A test of the influence of concentration upon the measured heat of transition as well as an independent check of the previous results were made in run IV which consisted of a freshly prepared solution of approximately half the concentration of that used in the preceding runs. The measured heat effect was proportionately smaller for this case indicating a negligible concentration effect. The values obtained in runs I, II, and IV serve to indicate the general consistency of the measurements. It is felt that the accuracy of the calorimetric result is of the order of ±1%. The measured heat of transition per mole of ribonuclease (mol. wt. 13,683) is therefore found to be 70 ± 1 kcal./mole^{-1}. The baseline shift prior to and after the transition reflected a change in heat capacity of 0.66 kcal./mole-deg. with an estimated error of about 10%. This change in heat capacity is an order of magnitude smaller than values reported for the acid denaturation of ferrihemoglobin (9.9 kcal./mole-deg.[13])

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Table II. Selection of Reported Enthalpies for the Ribonuclease Transition[a]

Method	pH	ΔH_1, kcal.	ΔH_2, kcal.	Source
Calorimetric	2.8	+52.2	...	This work
Sedimentation	2.8	+68	...	Holcomb[6]
Viscosity	2.8	+67	...	Holcomb[6]
Optical density	0.9–6.8	+51 (calculated below Tr)	...	Hermans[4]
Optical rotation	0.9–6.8	+51 (calculated below Tr)	...	Hermans[4]
Enzymatic activity	5	+35	...	Kalnitsky[5]
Differential optical density	2.9	+79.2	+26.5	Scott and Scheraga[7]

[a] ΔH_1 calculated from the slope of the equilibrium constant based on one-step reaction process. ΔH_2 obtained for second step in a two-step mechanism.

and serum albumin (8.0 kcal./mole-deg.[14]). However, the method and conditions for determining these latter heat capacity changes are quite different (*i.e.*, an acid shock) from our measurements on the thermal transition phenomena.

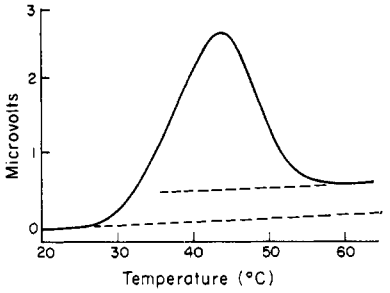

Figure 1. Recorder trace for the transition of run 1 (see Table I) with low and high temperature baselines drawn as dotted lines.

The solution used in runs I, II, and III was examined by gel electrophoresis[15] in order to determine if changes in the material might have been introduced upon the thermal treatment.

The disk pattern obtained for ribonuclease dissolved in buffer differed from patterns obtained for water solutions by the appearance of a wider spread band.[15] The solution which had been subjected to the two thermal runs (I, III) differed from an unheated buffered solution by the appearance of a fast moving minor component. An estimate of the portion of the initial protein represented in this minor fraction was made by densitometry of the stained electrogram and was less than 1% of the total protein. This evidence suggests that the state of the starting material is unaffected by being subjected to thermal transition when dissolved in the buffer used.

A calculation of the fraction of thermal denaturation at various temperatures is also possible from the heat of transition curves provided one assumes that the heat effect at a given temperature is proportional to a fraction of denaturation θ. A plot of the results of such a determination is shown in Figure 2 where the thermal curves obtained by Holcomb and Van Holde[6] are also presented for comparison. It should be noted that a smaller slope is found from the heat-calculated fraction curve than from either the viscometric or sedimentation determinations. One way of comparing these differences is to calculate the change in enthalpy (ΔH_1) for an assumed one-step equilibrium process in the region of $\theta = 0.5$. A tabulation of such values for the ribonuclease transition is given in Table II. It is

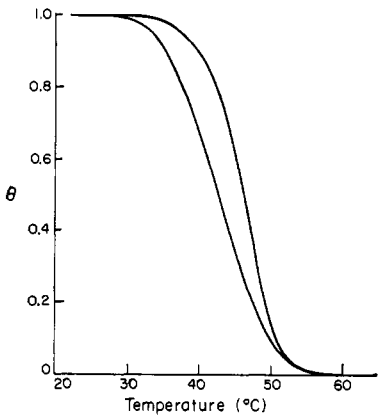

Figure 2. Fraction of transition, θ, as a function of temperature. Lower curve calculated from run 1, upper curve from the data of Holcomb and Van Holde.[6]

apparent that the value of ΔH_1 depends upon the method used to determine the fraction of denaturation. This means that the reaction is not a simple one-step process but must consist of at least two or more steps. Indeed, from a careful study of the transition by means of difference spectra Scott and Scheraga[7] concluded that the transition mechanism could be explained by a two-step reaction process with enthalpy changes for the two steps as indicated in Table II. Holcomb and Van Holde had also suggested this possibility as well as the possibility of a heat capacity change to explain the temperature dependence, but did not make a definite choice. The calorimetric value found for the transition is quite close to the ΔH_1 found by Holcomb and Van Holde, but we feel that this must be regarded as due primarily to the physical method for evaluating the fraction of denaturation. From the two-step analysis of Scott and Scheraga[7] the total heat of reaction is the sum of the two-step enthalpies given in Table II, and is *ca.* 107 kcal./mole. This value is far outside the range of error for the calorimetric value of 70 kcal./mole. The effect of concentration was negligible in both determinations, although the general range of concentrations differed by an order of magnitude. The two-step process determined by Scott and Scheraga was indicated by the resolution of the difference spectra into two first derivative overlapping peaks, with transition temperatures of 42.5 and 26.5° for the pH 2.9

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(15) R. A. Reisfeld, U. J. Lewis, and D. E. Williams, *Nature*, **195**, 194 (1962).

solution. From this result one would predict a calorimetric observation of two peaks, or at least a shoulder, in the e.m.f. trace of Figure 1. The lack of such a feature in Figure 1 suggests that the resolution accomplished by Scott and Scheraga might not be completely correct. In making the resolution it was assumed that the heat of each step was independent of temperature. We have noted a small but definite heat capacity change for the transition, which would be contrary to this assumption. We have found that the calorimetric fraction temperature dependence in Figure 2 can be explained by either a two-step simultaneous process or a two-step successive process for various combinations of heat of reaction per step and transition temperature. It is also possible to obtain correspondence with the curve in Figure 2 by helix–coil transition theory. For example, if the transition reaction consisted of successive steps which involve equal amounts of heat, then the fraction as determined by the heat measurements provides a significant parameter for determining the extent of the reaction. There is no special reason to believe that this situation holds in ribonuclease in contrast to simple synthetic polypeptides; indeed, some of the evidence points to the contrary. However, if one does go ahead and make this questionable assumption, then it is possible to apply the results of helix–coil transition theory[16] to the experimental dependence of θ with temperature. When this is done then one finds various combinations of parameters, σ and n, which can give theoretical curves that fit the experimental curve reasonably well. It is of interest in this connection to note that when one takes a σ-value of 2×10^{-4}, which has been found to apply to polypeptide transitions, that a value of n of 70, which corresponds to the estimated number of carbonyl–amide hydrogen bonds in ribonuclease,[17] does not give a theoretical curve that fits the experimental data. For this value of σ a much larger value of n would be needed for a reasonably fitting transition curve.

In conclusion the calorimetric results serve to confirm the notion that the ribonuclease transition is not a simple helix–coil polypeptide transition, nor is it a transition which consists of a single step or several independent steps with significantly different transition temperatures. The enthalpy measurements indicate the transition reaction consists of more than one closely affiliated or similar step.

Acknowledgment. This work was supported by Public Health Service Grant GM 08264-04.

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Kinetics of the Flavin Mononucleotide System

James H. Swinehart[1]

Contribution from the Max Planck Institute for Physical Chemistry, Goettingen, West Germany. *Received September 18, 1964*

The kinetics of the equilibria present in a partially reduced aqueous solution of flavin mononucleotide are examined by the temperature-jump–relaxation method. The equilibria considered are the formation of a dimer from the oxidized and reduced forms of flavin mononucleotide (eq. i) and the formation of free radicals from the dimer (eq. ii). (i) is established rapidly compared to (ii). In the pH range 3.9–5.2 at 11° and ionic strength 0.1 the rate constants for (ii) are $k_{32} = 4 \times 10^7\ M^{-1}\ sec^{-1}$ *and* $k_{23} = 0.8\ sec.^{-1}$. *For (i)* k_{21} *and* k_{12} *are greater than* $2 \times 10^5\ sec.^{-1}$ *and* $4 \times 10^8\ M^{-1}\ sec.^{-1}$, *respectively.*

Introduction

The species and equilibria present in partially reduced aqueous flavin solutions have long been a subject of interest. Kuhn and Wagner-Jauregg[2] reduced flavin dyes in the presence of HCl and noted a transition from the original intense yellow color to red and then to pale yellow, the color of reduced flavins. The red species was assumed to be a semiquinoid radical. Michaelis and his co-workers[3,4] made a detailed quantitative study of a number of flavin systems by analysis of potentiometric titration curves. At low concentrations of the flavin and over a wide pH range it was concluded that the intermediate form in the reduction is represented by a free radical. At higher concentrations of the flavin there is a partial dimerization of the free radical. Lowe and Clark[5] made potentiometric titration studies of several flavins over a wide pH range. They concluded that for flavin mononucleotide the amount of flavin as free radical and dimer was essentially constant over the entire pH range at a total concentration of $10^{-4}\ M$ in flavin.

Changes in the color of flavin solutions upon reduction were noted by early workers.[2-4] However, Beinert was the first to assign species to the absorption bands which appeared during the reduction of flavins.[6,7] Bands at about 570 and 900 mμ were assigned to the free radical and dimeric forms, respectively. A strong temperature and dilution dependence was noted for the longer wave length band. It was observed that a plot of the optical density of the 570 mμ band *vs.* the optical density of the 900 mμ band did not follow

(1) Address correspondence to the Department of Chemistry, University of California, Davis, Calif.
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