Volume 5 Number 5 May 1978 Nucleic Acids Research

Calorimetric studies on viroids

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Received 6 February 1978

ABSTRACT

Thermodynamic studies on highly purified viroid preparations were carried out with the help of a very sensitive adiabatic microcalorimeter. Parallel to the change of UV-absorption at 260 nm as a function of temperature, the additional heat capacity of the dilute viroid solution rises sharply within the melting interval, reaches a maximum at $T = T_m$ and declines to a baseline again when the temperature is increased further. From the peak area the molar transition enthalpy can be calculated. The transition enthalpies of citrus exocortis viroid and cucumber pale fruit viroid are 4200 kJ/mol and 3930 kJ/mol, respectively. The calorimetric results are compared to the results obtained from melting studies using UV-absorption.

INTRODUCTION

In the preceding paper [2] the thermal denaturation process of viroids was studied by thermodynamic and kinetic methods using UV-absorption. From those studies only apparent transition enthalpies could be experimentally determined because an all-or-none description was inadequate. An elaborate statistical mechanical treatment based on detailed model assumptions was applied to obtain the true transition enthalpies. Therefore, it would be highly desirable to use an independent method for direct determination of the true enthalpies. In this way additional support for the model of the secondary structure of viroids proposed in the preceding paper could be achieved. Calorimetric measurements can provide such an additional support because true transition enthalpies are experimentally determined without model assumptions if the molar concentration of the biopolymer is known. Using a newly designed scanning microcalorimeter for liquid samples [3] true transition enthalpies could be determined for very small quantities of highly purified viroids. From the experimental

curves the degree of transition as a function of temperature can be calculated similarly as from optically recorded melting curves, which leads to a value of $H_{van't\ Hoff}$. Applying standard thermodynamic relations, values for the cooperativity and the transition entropies can be obtained.

MATERIALS

Purification of CPFV [1] and CEV [1] and sample preparation were carried out as described in the accompanying paper [2].

METHODS

Calorimetric measurements were performed with an adiabatic differential scanning calorimeter, Type DASM, constructed by P. Privalov [3], Institute of Protein Research of the Soviet Academy of Sciences. The sample chamber was filled with viroids in a concentration of 0.1 - 0.2 mg/ml dissolved in 0.01 M sodium cacodylate pH 6.8, 1 mM EDTA. The same buffer was placed in the reference cell. The sample and blank were heated at a constant rate of 1.0°C/min. The additional energy required to keep the temperature difference of the two cells close to zero, was recorded as a function of temperature. The enthalpy change, due to the order-disorder transition of the viroids can be obtained by comparing the experimental peak area with a calibration mark put on the recorder tracing by an external electronic signal. The concentration of the solution was determined by measuring the optical absorption at 260 nm before heating. Optical melting curves were determined as described in [2].
The molar absorption coefficient was measured by a quantitative phosphorus determination [6]. Particular case was taken to avoid an interference of the cacodylate buffer with the phosphorus determination [7].

RESULTS

Molar absorption coefficient

The molar absorption coefficient was determind on CEV in 0.01M sodium-cacodylate pH 6.8, 1mM EDTA at 25°C. Based on a molecular weight of 119000 [8] the following result was obtained:

$$\varepsilon_{260} = 2.95\ (\pm 0.03)\ \ 10^6\ M^{-1}cm^{-1}$$

or $40.7(\pm 0.4)\ \mu g/\ A_{260}^{1cm}$ in 1 ml.

For CPFV the same absorption coefficient has assumed, because the physical properties of CEV and CPFV are very similar (cf. the accompanying paper [2]). It is interesting to note, that only a 1% lower absorption coefficient is obtained, if it was calculated from the absorption coefficients of all nucleotides [5] according to the nucleotide composition (H.J. Gross et. al., to be published) and a hypochromicity extrapolated to 100°C of 35% was taken into account.

Calorimetric measurements

Fig.1 and Fig.2 show the compensating energy as a function of the temperature for CEV and for CPFV. The peaks are almost symmetrical with a "tail" at the low temperature side. Repeated heating of the same sample leads to an increase of the lower melting part of the whole process (dashed lines in Fig. 2) while the total area of the peak stays constant. The transition at lower temperatures (T_m 48°C in CPFV) will be discussed together with the optical result.

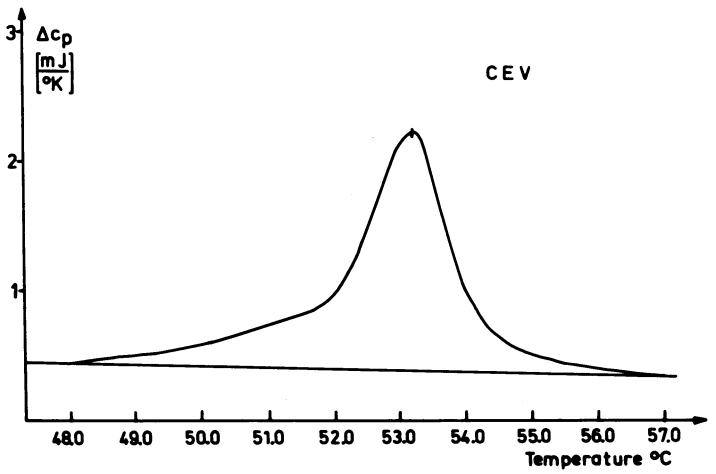

Fig. 1: Additional heat capacity as a function of the temperature for CEV. Heating rate 1°C per min.

The T_m-value of the major transition in CPFV agrees with the value determined in optically recorded melting curves. The T_m-value of CEV is about 2.5°C higher than the optically determined one. This is most probably due to a slight difference in the ionic strength of the sample, since the samples were not dialysed prior to the experiments.

In Table 1 the results from the calorimetric measurements

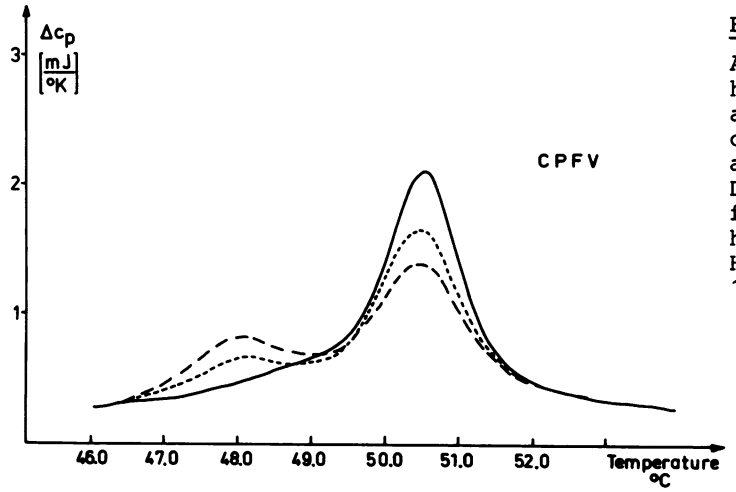

Fig. 2:
Additional heat capacity as a function of the temperature for CPFV. Dashed lines for repeated heating. Heating rate 1°C per min.

are listed. The temperature at the maximum of the compensating energy peak was taken as T_m. ΔH-values were determined from the peak areas which were evaluated by comparing them to a known calibration peak. $\Delta H_{van't\ Hoff}$ was calculated from the plot of the degree of transition vs. temperature as outlined by Breslauer, Sturtevant, and Tinoco [4]. From standard thermodynamic equations ΔS can be determined at $T = T_m$ under the assumption that the equilibrium constant of the order-disorder transition equals unity at T_m.

TABLE 1

Viroid	T_m |°C|	ΔH |kJ/mol|	$\Delta H_{van't\ Hoff}$ |kJ/mol|	ΔS |e.u.|
CPFV	50.5±0.2	3930±150	2680±150	2920
CEV	53.1±0.2	4200±150	2630±150	3090

The error limit of ΔH includes the experimental inaccuracy of the calorimetric measurement and of the molar extinction coefficient.

Optical melting curves

Optical melting curves have been carried out to clarify the features of the transition at lower temperatures, which appeared after repetitive calorimetric scanning of CPFV. The sample of CPFV after the fourth cycle has been transferred to the UV-absorption cuvette and in a fifth cycle the melting curve was recorded optically (Fig.3). As seen from Fig.3 the low temperature transition showed up very similar in the optically recorded melting

curve as expected for a repeated calorimetric curve. Evidently, the low temperature transition is induced by an irreversible change of the viroid, detectable in both types of melting curves. The width of the main transition is similar to the width recorded on samples in which no low temperature transition had appeared, e. g. in the first calorimetric cycle or in melting curves of the accompanying paper [2]. Therefore, we conclude that the main part of molecules remains unaffected and that the low temperature transition belongs to a different molecular species. Most probably, some viroids are split at a single side, which means that the circular chain is converted to a linear chain. It is shown in the accompanying paper that the melting temperature is expected to be some degrees lower. Also the cooperativity in split molecules is expected lower, and should depend upon the position of splitting which cannot be derived from the present experiments. The chain splitting of some viroids of the sample may have been due to a nuclease activity in the sample or due to a heavy metal ion impurity. We regard the second possibility as more probable, since the method of purification of viroids, certainly, resulted in nuclease free samples as is seen from the studies of the accompanying paper.

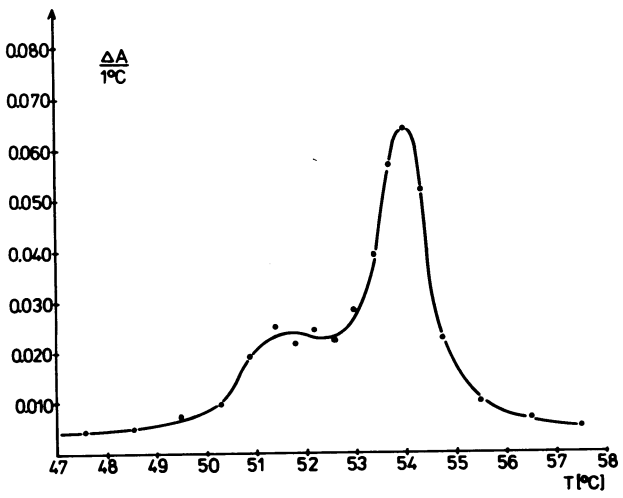

Fig. 3: Optically recorded differential melting curve at 260 nm of the sample of Fig. 2. A (260 nm, 20°C) = 1.

DISCUSSION

The ΔH-values determined on CPFV and on CEV are similar and their limits of error are overlapping. Thus, the thermodynamic similarity of both viroids are evident from these studies as well as from the optical studies [2]. As pointed out in the results, slight variations in T_m may be caused by differences in the ionic strength. One has to take into account, that the sample size was only about 100 µl or less, furthermore, that the samples were degassed and heated prior to the experiment, run through several heating cycles during the measurement and were transferred from the calorimetric to the optical cuvette. All these procedures had been carried out without a dialysis in between to avoid any loss of material. Therefore, the T_m-values determined calorimetrically or optically are not at variance.

The calorimetric value of ΔH represents the total enthalpy of the order-disorder transition in viroids. Its value is independent upon any assumption of the secondary structure of viroids. The conclusions of the preceding paper which are based on optically recorded thermodynamic studies and statistical thermodynamic analysis are in accordance with the present calorimetric data. For CPFV a calorimetric value of 3930 kJ/mol compares to a total enthalpy from optical studies of 4080 kJ/mol, and for CEV 4200 kJ/mol to 3990 kJ/mol.

The values of $\Delta H_{van't\ Hoff}$, a measure for the cooperativity of the transition, are smaller than the apparent reaction enthalpies determined by the optical methods. In part, the difference is understandable, since the apparent reaction enthalpies were corrected by kinetic means for the uncooperative single strand destacking. The remaining difference is obvious from a close inspection of the calorimetric and the optical melting curve, showing a smaller halfwidth of the optical melting curve. This difference which may be due to a lag of the calorimeter was not investigated in more detail. It does not affect, however, the accuracy of the total reaction enthalpy.

From the total enthalpy 85 base pairs (with considerable uncertainty) were deduced to exist in the native structure in a series of helical sections and internal loops. It was shown that the number of base pairs may not be obtained in a straight for-

ward manner because of the pecularities of the secondary structure. The calorimetric studies support the conclusions on the secondary structure to the extent that they confirm experimentally the high cooperativity deduced from the model, they cannot, however, circumvent details in the assumption on the size and distribution of helical sections and internal loops.

ACKNOWLEDGEMENT

We thank Mrs. C. Strasser for the quantitative phosphorus analysis, Mr. O.E. Beck and Mr. K. Henco for critically reading the manuscript. The calorimetric experiments were carried out during a stay of H.K. in the laboratory of Dr. P. van Hippel, U.o.O., Eugene, Oregon. The calorimeter was purchased from grant No. GB 29709 from the National Science Foundation. The work was supported by the Deutsche Forschungsgemeinschaft (SFB 47 and personal grants).

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