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Conformation of viroids

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ABSTRACT

Viroids are uncoated infectious RNA molecules (MW 107 000-127 000) known as pathogens of certain higher plants. Thermodynamic and kinetic studies were carried out on highly purified viroid preparations by applying UV-absorption melting analysis and temperature jump methods. The thermal denaturation of viroids is characterized by high thermal stability, high cooperativity and a high degree of base pairing. Two relaxation processes could be resolved; a process in the sec range could be evaluated as an independent all-or-none-transition with the following properties: reaction enthalpy = 550 kcal/mol, activation enthalpy of the dissociation = 470 kcal/mol; G : C content = 72 %. These data indicate the existence of an uninterrupted double helix of 52 base pairs. A process in the msec range involves 15 - 25 base pairs which are most probably distributed over several short double helical stretches. A tentative model for the secondary structure of viroids is proposed and the possible functional implications of their physicochemical properties are discussed.

INTRODUCTION

The term viroid has been used in recent years to describe minimal-sized infectious RNA molecules which behave biologically very similarly to conventional viruses. These RNA molecules are the agents of several economically important diseases of certain higher plants (see Review by T.O. Diener [1]).

In contrast to the RNA of usual viruses, viroid RNA is not protected by a coat protein. Although this does not exclude a possible viroid protein complex inside the host cell, infectivity and pathogenicity are associated with viroids isolated as protein-free RNA molecules. The second striking feature of viroids is their low molecular weight of about 120 000 daltons, i.e. the RNA is about ten times smaller than the RNA of the smallest known bacterial viruses such as phage MS 2 or R 17. Since the potential genetic information is not sufficient to

code for a protein with a molecular weight larger than 10 000 daltons, viroids cannot directly code for a viroid specific RNA polymerase. Accordingly little is yet understood about their mechanisms of replication and pathogenicity.

During the last two years three groups have succeeded in purifying viroid RNA in amounts sufficient for physico-chemical investigations [1, 2, 3] .

Several lines of indirect evidence led Diener [1] to the conclusion that viroids may be either single-stranded RNA molecules of hairpin structure and extensive base pairing or double-stranded RNAs with incomplete base pairing. From optical and NMR investigations Semancik et al. [2] inferred that viroid RNA is highly structured, and that 70 - 80 % of all base pairs are G : C pairs. In a recent study using hydrodynamic, thermodynamic, electron microscopic, and biochemical methods, we described several new structural features of the viroid molecule [3]. First, by equilibrium sedimentation the molecular weights of six different viroids were determined to range between 107 000 and 127 000 daltons. Secondly, it was shown that viroids exist as a single nucleotide strand, which is partially refolded and assumes a rod-like shape in its native conformation. Finally, and most surprising, electron microscopic studies on denatured viroids, and viroid RNA endgroup analysis revealed that viroids are covalently closed circular molecules.

In the present work we report physico-chemical investigations on two viroids, CEV [4] from tomato and CPFV [5] from tomato. From quantitative thermodynamic and kinetic data on the thermal denaturation of viroids relevant conclusions about the secondary structure can be drawn. We provide evidence for the existence of distinct structural regions in viroid molecules which may be responsible for specific biological functions.

MATERIALS

The purification of the viroids CEV [4] and CPFV [5] , both from tomato, up to gel electrophoretical homogeneity has been described in detail elsewhere [6]. According to the analysis under native and denaturing conditions in gels of different acrylamide concentration their homogeneity was estimated to be

higher than 99 %. The final alcohol precipitates were washed twice with ethanol, dissolved in triple-distilled water and used as stock solution without further dialysis.

The standard buffer was 0.01 M sodium cacodylate, 1 mM EDTA, pH 6.8. All chemicals were of analytical grade, and buffer solutions were made up from triple-distilled water. Melting curves in standard buffer (see below) were identical, independently of whether 1 mM or 0.1 mM EDTA was present; therefore, possible contaminating divalent cations had been complexed completely by EDTA, and did not affect the measurements. Before the measurements all samples were heated for 5 minutes up to 70 ^{o}C and cooled down slowly. After this treatment viroids were highly infectious as shown in the bioassay [6] .

METHODS

Differential Melting Curves

Melting curves have been measured by the differential absorption technique as described earlier [7] . According to the steepness of the transition the difference in the temperature between the two cuvettes had to be smaller than 0.3 ^{o}C.

Relaxation Kinetics of Processes faster than 5 sec

An improved version [8] of the Eigen/DeMaeyer temperature jump apparatus was used. The instrument was equipped with a high intensity double monochromator (2 x Schoeffel G 250, 3.3 nm/mm dispersion, blazed at 240nm, 1 mm slit width). The high dispersion and spectral purity were relevant for the accurate measurement of the wavelength dependence of the relaxation amplitudes. The cell was particularly selected to be free of schlieren up to 10 sec after the temperature jump. This condition was fulfilled best by a cell-type in which the central part is made from fused polished pieces of quartz [8] .

Relaxation Kinetics of Processes slower than 3 sec

Slow relaxation processes have been studied by the method of Pohl [9] . The temperature of the sample was raised or lowered from T_1 to T_2 within 0.5 sec by switching the thermostating circuit from one bath with the temperature T_1 to another with the temperature T_2. The design of the cuvette was also similar

to that of Pohl [9] but it was particularly adapted for the use at high temperatures. The details of the design and operation will be described elsewhere (Henco and Riesner, in preparation).

The cell was adapted to a ZEISS DMR 22 spectrophotometer equipped with a 200 Watts Xenon-Mercury Lamp (Hanovia). The time course of the change in absorption was stored on a digital oscilloscope (Data Lab.) with external time base. The temperature of the sample was determined by a thermistor in the cooling solution immediately adjacent to the outflow from the cell.

Evaluation of the Kinetic Measurements and Cooling Correction

The evaluation of the oscilloscope traces has been carried out by an analog simulation technique as described [10] .

As will be seen from the results it was essential to follow the relaxation times continuously from the time range of msec to min. The range of relaxation times between 1 and 5 sec is scarcely experimentally accessible. Those relaxation times are very fast for measurements with the slow T-jump method; in the fast relaxation technique, however, the influence of cooling of the solution which proceeds in this time range affects the relaxation processes remarkably. This influence has been corrected by solving the differential equation of the perturbation in concentration x_A for a monomolecular process $A \underset{k_D}{\overset{k_R}{\rightleftharpoons}} B$ and taking into account the temperature dependence of the constants k_R and $K = k_R/k_D$ and of the deviation of the equilibrium concentration $\Delta\bar{c}_A$ from the concentration before the jump. It can be verified easily 23 :

$$\frac{dx_A(t)}{dt} = -k_R(t)\ (1+\frac{1}{K(t)})\ (x_A(t) - \Delta\bar{c}_A(t)) \ .$$

The time course of the cooling was determined experimentally.

RESULTS AND DISCUSSION

I. Equilibrium Measurements

The equilibrium measurements serve to give a graphic description of the melting process, whereas the quantitative values will be evaluated exclusively from the kinetic data (see below).

The thermal denaturation profiles were determined by UV absorption melting curves.

Melting Curves at Standard Conditions

Standard buffer conditions of low ionic strength (0.01 M sodium cacodylate pH 6.8, 1 mM EDTA) had been chosen to obtain melting temperatures below 60 °C. In Fig. 1 the melting curves of CEV and CPFV are presented in the integrated form (a) and the differentiated form (b). The striking feature of the viroid melting is the narrow temperature range in which the whole denaturation occurs. It may be seen most easily in the differentiated melting curve that the melting process is highly cooperative. For comparison, melting curves of $tRNA^{Phe}$ (yeast) obtained under identical conditions have been added to the figures. These curves exhibit the well known melting of tRNA [8,11] which is spread over a temperature range of more than 40 °C,

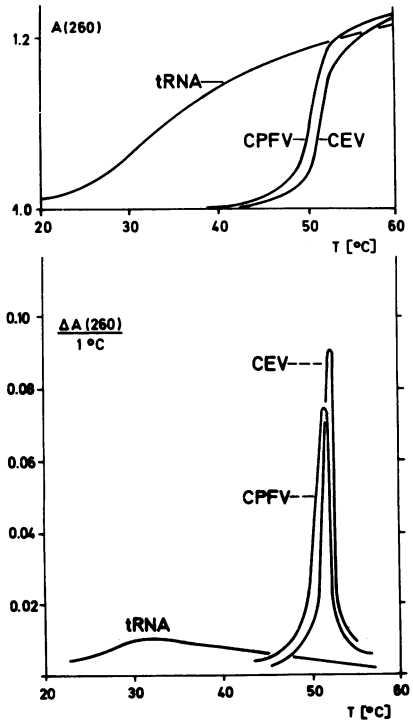

Fig. 1:
Melting curves of CEV, CPFV and $tRNA^{Phe}$ (yeast) under standard conditions in the integrated form (a) and differentiated form (b). A (260 nm, 20°C) = 1 .

reflecting the more or less sequential process of the melting of the different branches of its clover-leaf structure. To our knowledge such a steep melting as that shown by viroids has not been observed with any other single stranded natural RNA hitherto. When the total hypochromicity was determined either directly or from the area below the differential melting curve, a value of 22 % was obtained at 260 nm. This value is similar to those known from tRNA, 5S RNA or 6S RNA and indicates a comparable amount of base pairing.

Influence of Urea

As expected for nucleic acids the addition of urea lowers the melting temperature (Fig. 2). It is interesting to note that the shape of the melting curve remains essentially the same up to 8 M urea, whereas the hypochromicity decreases continuously with increasing concentrations of urea. This decrease is most probably an indirect consequence of the presence of urea. Since the degree of stacking of the single strands is increased at lower temperature, the difference in the extinction coefficients between the double-helical and the single-stranded state is smaller during the melting at low temperature, as compared to the melting at higher temperature. The knowledge of

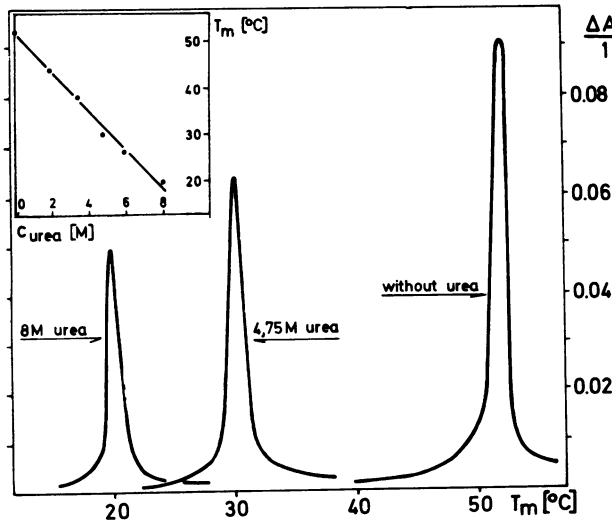

Fig. 2: Influence of urea on the melting curve of CEV. Inset: Dependence of melting temperatures of CEV on urea concentration.

the melting behaviour of viroids in the presence of different concentrations of urea was applied during their purification procedure. In 8 M urea at 15 °C, for example, viroids are still double-helical, whereas all host RNA is at least partially denaturated. Therefore, host RNA which will comigrate in the gel with viroid RNA under non-denaturing conditions may be separated easily from it in the presence of 8 M urea [6] .

Minimum Value of the Reaction Enthalpy ΔH

The reaction enthalpy of the melting process can be used to estimate the number of base pairs which open up in the denaturation process. As a first order approximation one may treat the melting process as an interconversion between only two states assuming that the molecules may form either the maximum number of base pairs or may be completely single-stranded. The degree of transition and the equilibrium constant are taken from the width of the total melting curve [11] . A reaction enthalpy of 490 ± 30 kcal/mol was obtained. This value can be regarded only as a lower limit, since any deviation from the two state model will result in higher values.

II. Kinetics

The mechanism of the denaturation process was studied by kinetic measurements with the temperature-jump method.

1. Resolution of different relaxation processes

Fig. 3 shows an oscillogram which is obtained from a fast temperature-jump experiment. The first 10 msec of the curve are given with an extended time scale and a higher amplification of the intensity in the inset. It is demonstrated that at least three different relaxation processes are present:

The unresolved relaxation process occured simultanously with the temperature increase; this process could not be resolved on the time scale even if the heating time had been lowered to 6 μsec using a smaller capacitor for a faster discharge. The process is mainly due to the well known stacking-destacking equilibrium of single stranded regions of nucleic acids [11] which can occur in less than 1 μsec [12] . There is also a

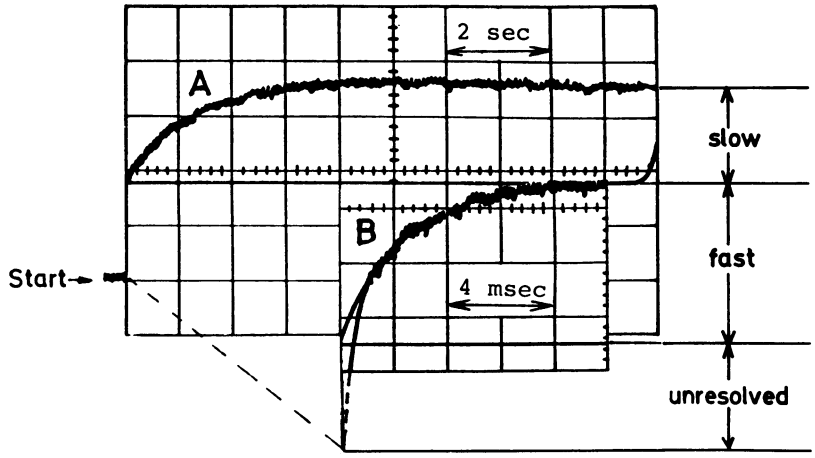

Fig. 3:
Oscilloscope traces of one temperature jump showing three kinetically resolved relaxation processes. Final temperature 52.3°C, $\Delta T = 1^{o}$ C, 280 nm.
A: Slow denaturation process with τ = 1.4 sec; oscilloscope settings: 1 sec/unit; 50 mV/unit; total signal: 3 V; 100 µsec noise filter; pretrigger.
B: Fast denaturation process with τ = 2.2 msec and the unresolved effect. Oscilloscope settings: 2 msec/unit; 20 mV/unit.

contribution due to the temperature dependent extinction coefficient of EDTA which has been subtracted in all further treatments.

The fast relaxation process could be described by a relaxation time in the msec range. It was well separated from the unresolved and from the slow process. At present, it cannot be excluded that more than one relaxation process with relaxation times differing by less than a factor of three are hidden under the fast process, but there is no experimental indication for this. It will be shown that short double helices are responsible for the fast relaxation.

The slow relaxation process in the sec range represents the main contribution of the total change in hypochromicity. If its relaxation time was shorter than 3 sec it could be followed only in fast temperature jump experiments, if longer than 10 sec only the slow temperature jump method could be applied.

Obviously, this relaxation represents a major conformational change involving many base pairs.

2. Relaxation amplitudes

The amplitudes of the separated relaxation effects can be used to evaluate a differential melting curve not only for the total hypochromic effect but also for each resolved process separately. As described by Riesner et al. [13] , the best accuracy is achieved if the total hypochromic effect is taken from equilibrium measurements $(\Delta A/\Delta T)_{equ}$ and the ratio of the amplitude A_τ of the resolved process with the relaxation time τ to the total relaxation process A_{total} from kinetic measurements. In this way the differential melting curve of a process with the relaxation time is defined:

$$\left(\frac{\Delta A}{\Delta T}\right)_\tau = \frac{A_\tau}{A_{total}} \cdot \left(\frac{\Delta A}{\Delta T}\right)_{equ}$$

Temperature dependence. In Fig. 4 the temperature dependence of the amplitudes of the total and the resolved relaxation processes is given. Only the slow effect exhibits the marked dependence upon the temperature shown in the melting curve in Fig. 1. The fast relaxation process can best be described by the superimposition of a narrow peak over a very broad background. It follows that this process cannot be regarded as one

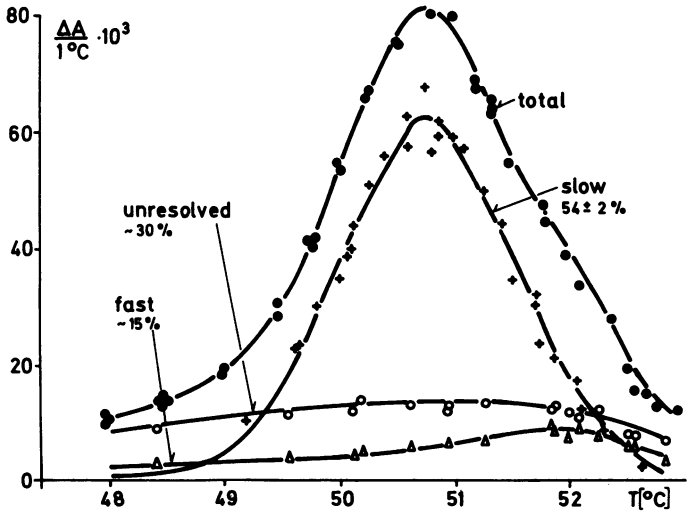

Fig. 4: Separation of the total melting curve in three distinct denaturation processes.

independent homogeneous transition; although it amounts in total only to 25 % of the slow process, its amplitudes are larger than those of the slow process at low temperatures and at high temperatures.

The slow process was evaluated on the basis of an all-or-none-transition yielding a straight line in the van't Hoff plot and a reaction enthalpy of 500 ± 50 kcal/mol (Fig. 5).

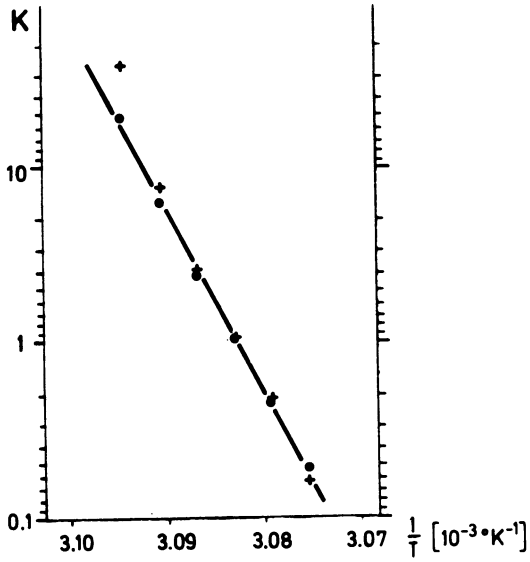

Fig. 5:
van't Hoff plot evaluated from the amplitudes of the slow denaturation process of CPF-Viroid.
+ measured at 274 nm
· measured at 280 nm

Wavelength dependence. The wavelength dependence of the hypochromicity is different when AU base pairs or when GC base pairs are melting. It has been shown by Fresco and coworkers [14] that the spectrum of the hypochromicity of a melting double-helix with an inhomogeneous sequence can be superimposed with good accuracy from the spectra of melting A : U pairs and melting G : C pairs. If the superimposition does not work this would be a strong indication for the existence of non Watson-Crick base pairs.

The spectrum of the hypochromicity has been determined from the relaxation amplitudes for the fast and the slow processes sepa-

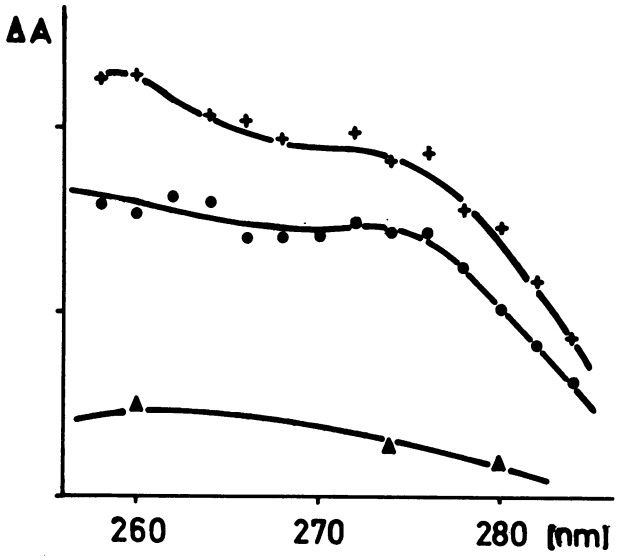

Fig. 6:
Wavelength dependence of the hypochromicities of the different melting processes.
ΔA in relative units,
+ ΔA total, · ΔA slow,
▲ΔA fast.
Mean error limits ΔΔA are about 4 %.

rately (Fig. 6). The spectrum of the slow process has been measured at several temperatures and gave within the limits of error always the same result. This is consistent with an all-or-none-process, in which the same molecular process occurs over the whole temperature range of the transition. The spectrum of the fast process has been followed only at the temperature of its maximum amplitude.
The spectra given in Fig. 6 are well superimposable from the known spectra [14] of A : U and G : C pairs, respectively. The decomposition of the measured spectrum showed that the slow process consists of 72 ± 2 % G : C and 28 % A : U pairs, whereas the fast process contains about 50 % G : C and 50 % A : U pairs. The significant difference in the composition of the fast and slow process demonstrates that distinct regions of the molecule correspond to the different relaxation processes.

3. Relaxation times

Characteristic features of the denaturation mechanism could be obtained from systematic studies of the relaxation times.

Concentration dependence. The concentration of viroid has been varied by a factor of three without resulting in any measurable change in the relaxation times. This result was expected, since all other data including molecular weight determinations did

not give any indication for the participation of a second order process under the conditions used in this work.

Dependence upon the height of the temperature-jump. These relaxation experiments are in one respect different from normal temperature-jump experiments. Because of the very steep melting curve the perturbation of the equilibrium is not always small compared to the absolute concentrations. A temperature jump of 0.5 °C corresponds at T_m to 30 % of the whole transition. It is, however, very easily verified that the time course of a first order reaction is not affected by the extent of the perturbation and the expression for the relaxation time $1/\tau = k_R + k_D$ [15] can also be applied for large perturbations. Experimentally, temperature jumps of different heights resulted in the same relaxation time if the final temperature was the same.

Temperature dependence. The relaxation times have been followed over the whole temperature range of the transition. In Fig. 7 the relaxation times are presented in form of an Arrhenius-plot. Whereas the fast relaxation time does not depend upon the temperature significantly, the measured values of the slow relaxation time vary continuously with the temperature over more than three orders of magnitude. These relaxation times have been

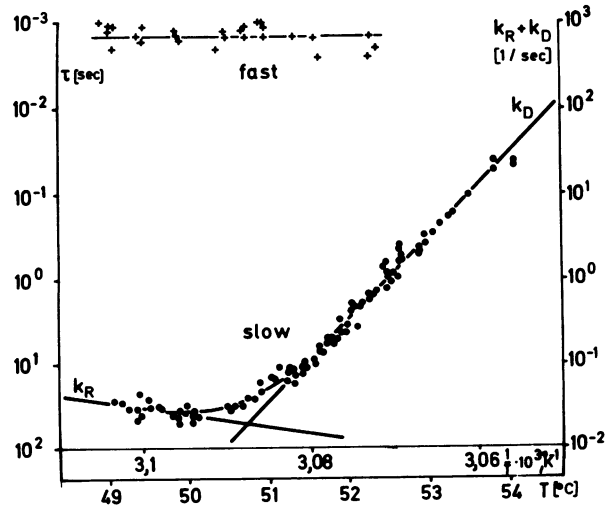

Fig. 7: Arrhenius-plot for the slow and the fast denaturation process.

fitted to the reaction scheme of one all-or-none-process according to $1/\tau = k_R + k_D$ (cf. Fig. 7), and an excellent fit was obtained for the whole range.

4. Activation- and reaction-enthalpies and number of base pairs

First, from the slope of the high temperature part of the Arrhenius-plot the activation enthalpy for the dissociation $E_D^{\ddagger}$ was evaluated with good accuracy:

$$E_D^{\ddagger} = 470 \pm 10 \text{ kcal/mol}$$

The extrapolation of k_D to lower temperatures and subtraction from $1/\tau$ leads to the value of the activation enthalpy of the recombination reaction $E_R^{\ddagger}$:

$$E_R^{\ddagger} = -80 \pm 30 \text{ kcal/mol}$$

The difference of $E_D^{\ddagger}$ and $E_R^{\ddagger}$ is the reaction enthalpy ΔH :

$$\Delta H = E_D^{\ddagger} - E_R^{\ddagger} = 550 \pm 40 \text{ kcal/mol}$$

This value of ΔH is obtained only from relaxation times. The value derived in a completely independent way from the amplitudes was 500 ± 50 kcal/mol and is in good agreement. So far, all sets of data are consistent with each other and consistent with an all-or-none-process.

From the value of the reaction enthalpy ΔH and the G : C/A : U-content it is possible to estimate the number of base pairs which open up in a cooperative melting process. For this estimation the values of ΔH per A : U pair and ΔH per G : C pair have to be known. Two sets of data are available from the literature. In the first one, given mainly by Pörschke et al. [16], the temperature dependence of the ΔH-values is accounted for while the influence of the sequence has been ascribed only to entropic effects; the second type of treatment [17,18] takes into account sequence effects, but does not describe the influence of the temperature. The different treatments may result

in large differences, if ΔH-values of particular sequences are calculated for lower temperatures. Fortunately, both methods give very similar results at 50 °C, the melting temperature of the viroids in this work. For the reason of highest simplicity we use the values of Borer et al. [17] ; it should be noted that these values were calculated partially from studies at similar temperatures as in our studies.

The complete cooperativity of the relaxation effect indicates that a regular, uninterrupted double helix is dissociated. We assume that the determined 72 % G : C pairs and 28 % A : U pairs form a statistical sequence.

The ΔH - value of each possible pair of nearest neighbours (e.g. $\Delta H \left(\begin{smallmatrix} A & A \\ U & U \end{smallmatrix}\right)$ = - 8.2 kcal/mol; $\Delta H \left(\begin{smallmatrix} G & G \\ C & C \end{smallmatrix}\right)$ = - 13.7 kcal/mol; $\Delta H \left(\begin{smallmatrix} A & G \\ U & C \end{smallmatrix}\right)$ = - 5.9 kcal/mol) is multiplied by its statistical weight and an average value of ΔH per base pair = 10.5 kcal/mol is calculated for the slow relaxation process of CPF viroid. It follows, that 52 ± 5 base pairs are involved in the slow cooperative helix coil transition. If the assumption of a statistical sequence is not valid, an additional error of about 15 % may be possible for either a highly clustered or a highly alternating sequence.

The negative activation enthalpy (-80 kcal/mol) points to a fast preequilibrium preceeding the rate limiting step of the recombination. Such a mechanism of recombination is well known and has been found in other synthetic and natural nucleic acids [11,19] . In this case between 4 and 9 base pairs have to form a fast preequilibrium. It should not be concluded, that the rate limiting step could be localized at a definite base pair; the preequilibrium may be fairly inhomogeneous and the measured value of the activation enthalpy may be regarded as an average value involving different states.

It has been pointed out earlier, that the fast relaxation process is not an independent homogenous transition. Therefore, the number of base pairs cannot be calculated from a van't Hoff plot. The comparison of the integrated hypochromicities of the

fast and the slow process results in a rough estimate of 15-25 base pairs. This value is associated with considerable uncertainty, since the hypochromicity of short double helices depends upon the length of the double helical segments and most probably several short double helices are involved in the fast relaxation process.

III. Structural and Functional Implications

The data in this work have shown that in viroid molecules at least two structural domains with rather different physical properties exist. The two conformational transitions which have led to that picture, have been assumed to occur independently of each other. This is to some extent an approximation since double helical regions within one circular nucleotide strand are thermodynamically coupled [11,20] .

However, the slow process involving the larger region behaves as an independent transition, whereas the fast process could not be evaluated appropriately in that manner. It seems reasonable that the relative influence of the large double-stranded region on smaller hairpins may be important, whereas on the other hand small hairpins would have only minor influence on the helix-coil transition comprising as many as fifty base pairs.

The structural features which emerge from the thermodynamic data are visualized in a rough model (Fig. 8). The slow, highly cooperative transition strongly supports the existence of a regular double helical stretch of about 52 base pairs. Very few mismatches which would not destroy the cooperativity cannot be excluded. The hypochromicity of this transition at 260 nm is about 11 % of the total absorbance at 20 ^{o}C. Although it is fairly inaccurate to estimate number of base pairs only on the grounds of hypochromicity data from synthetic polynucleotides [14], one would estimate some what more than 52 base pairs from the hypochromicity. The double helical stretch is responsible for the extended overall shape of the native molecule as revealed in hydrodynamic and electron microscopic studies [3] . It represents the rigid core of the molecule; its thermodynamic stability is based on the high G : C content of 72 % and the ring

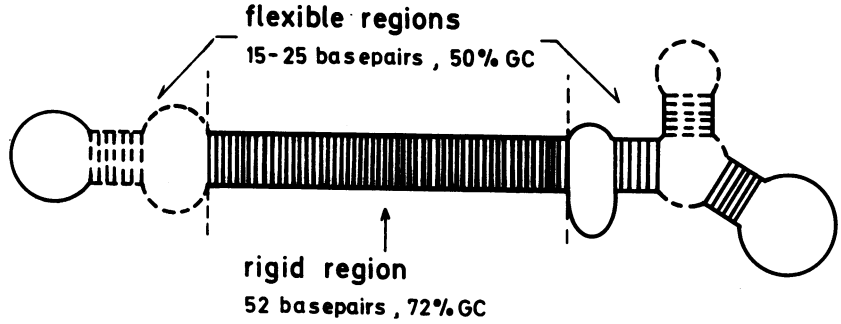

Fig. 8:
Model of a possible secondary structure of CPF-Viroid.

structure of the whole molecule [20] . Also the cooperativity of the slow transition is favoured by the ringstructure; this effect is known from the comparison of oligonucleotide hairpins and -circles [20] .

The fast relaxation effects show that also rather short hairpins are present. Since 15 - 25 base pairs are estimated to participate in this equilibrium and the equilibrium is still rather fast and independent of temperature, it would seem more reasonable to interpret these data by assuming the existence of several short hairpins instead of one regular hairpin containing all 15 - 25 base pairs. It is rather unlikely that the short hairpins are formed along the main double helix, since in that case the main double helix would be interrupted by internal loops and would loose its cooperativity. In summary, the viroid seems to contain a rigid DNA-like part and a more flexible tRNA- or mRNA-like part of the structure.
Both parts may fulfil quite different functions in replication and pathogenicity. A different dynamic behaviour is inferred from the thermodynamic and kinetic results. The stable part of the structure is present in the double helical conformation and has only a negligible probability of opening under physiological conditions. But the reflexible part of the structure is able to form significant concentrations of dissociated conformations, which have a lifetime of msec.
The presence of a tertiary structure remains an open question. Since for experimental convenience, the experiments in this

work had to be carried out in the absence of Mg^{++}-ions and at low ionic strength, it is possible that higher concentrations of mono- or divalent ions would induce an additional structure. It would be expected, however, that a tertiary structure if it exists would embrace preferentially the "tRNA-like" part of the molecule, since the extended overall shape has also been found experimentally in the presence of Mg^{++}-ions and 0.1 M NaCl [3] .

Structurally viroid RNA seems to have little in common with any of the known cellular or viral RNAs. However, one interesting candidate for structural and functional comparison is the Qβ RNA - fragment MDV-1 RNA [21] because of its comparable size and its capability of replication. But, although detailed physical data on MDV-1 RNA are lacking, few structural similarities would be expected on the basis of its known primary sequence and the multibranched secondary structure proposed [21] . Some features of the viroids are similar to those of an RNA described by Biebricher and Orgel [22] which had been obtained by selection from random copolymers, and which could be replicated by DNA-dependent RNA polymerase. This RNA is about half the size of the viroid, is thermally fairly stable despite a high content of A : U base pairs, and melts in a very cooperative way. Among other factors a regular double helical structure is most probably responsible that this RNA is replicated by DNA-dependent RNA polymerase. In this context it is tempting to speculate that the double helical stretch in viroid RNA might be of importance for viroid replication by a preexisting host cell system.

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