RESOLUTION OF FIVE CONFORMATIONAL TRANSITIONS IN PHENYLALANINESPECIFIC tRNA FROM YEAST

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1. Introduction

Recent progress in understanding the conformational dynamics of nucleic acids has been achieved by thermodynamic and kinetic studies of synthetic oligomers [1–3] and defined fragments of tRNA [4–6]. In naturally occurring nucleic acids, however, more complicated structures, including several base paired regions, seem to be important. Thus, we extended our studies to whole tRNA molecules [7, 8]. In $tRNA^{Ala}$ (yeast) four conformational transitions could be detected by differential UV absorption techniques. A similar analysis of $tRNA^{Phe}$ (yeast) seemed promising only after determining the melting behavior of the separated and recombined half molecules isolated from $tRNA^{Phe}$ (yeast) [4]. In addition, it was hoped that the unknown fluorescent Y-base in the anticodon loop of $tRNA^{Phe}$ (yeast) would facilitate the assignment of observed conformational changes to structural elements in this molecule. As a result of these studies we are now able to characterize five steps in the melting process of $tRNA^{Phe}$ (yeast). Furthermore, the coupling relations between these five transitions will be reported.

2. Materials and methods

$tRNA^{Phe}$ (yeast) was generously given by Dr. F.von der Haar and Dr. E.Schlimme. The sample preparation and the recording of the differentiated melting curves were as described in [7]. Fluorescence measurements were carried out in a Zeiss Spectrofluorimeter equipped with two monochromators and Polacoat-UV-polarizing filters. The fluorescence cuvettes were thermostated in the same way as in the absorption photometer, so that differentiated fluorescence melting curves could also be recorded. Relaxation kinetics were measured with the temperature jump method, the special modifications of which are described in [8]. Sedimentation coefficients $S^o_{20,w}$ were determined using a Spinco model E analytical ultracentrifuge equipped with an electronic multiplexer and a photoelectric scanner.

3. Results and discussion

Fig. 1 represents the differential melting profile of the complete $tRNA^{Phe}$ (yeast) molecule in the absence of Mg^{2+}. Above 60° this curve coincides with the sum of the melting curves of the two halves. In addition, comparisons of the difference spectrum ratios $\Delta OD^{260}/\Delta OD^{280}$, and of the temperature jump relaxation curves strongly indicate that above 60° the secondary structure of the complete tRNA is the same as that of the isolated rT- and hU-branches. The assignment of the remaining section of the melting curve is more difficult. As compared to the recombination peak of the half molecules, this part of the melting curve is shifted to higher temperatures. This shift may be attributed to the much higher effective concentration of the halves in the unsplit tRNA, i.e. the smaller average distance between the complementary nucleotides in the non-base-paired coil states. In the lower part of the melting profile (between 20° and 30°) the difference spectrum ratios decrease from 3.1 to 1.6. Assuming two two-state transitions for this part of the melting process, one may derive the two corresponding
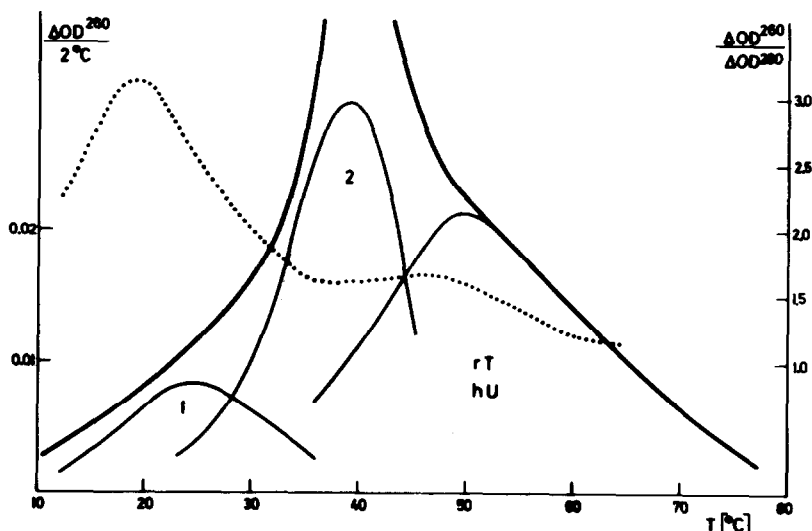
Fig. 1. Differential melting curve of tRNA^{Phe} (yeast) in 0.01 M sodium cacodylate, 0.02 M NaCl, pH 6.8. *Heavy solid line*: experimental values of $\Delta OD^{260}/2°$; *dotted line*: experimental difference spectrum ratios $\Delta OD^{260}/\Delta OD^{280}$; *thin solid lines*: transition curves of peak 1 and 2 as derived from the difference spectrum ratios and melting curve of rT- and hU-stem measured with half molecules [4, 5].

transition curves from the difference spectrum ratios [7]. The result is represented as peak 1 and 2 in fig. 1. Superimposing peak 1 and 2 as one coupled effect leads to a peak with the same values of hyperchromicity, difference spectrum ratios, reaction enthalpy as found in the recombination of the two halves. This finding indicates that the structures of the recombined halves and of the whole tRNA molecule are very similar, whereas the coupling of the different melting processes is drastically changed.

Changes of this kind may also be brought about by varying the experimental conditions, such as the concentration of mono- and divalent cations. Usually, minor changes of the reaction enthalpy ΔH are sufficient to produce 30° to 40° shifts of the transition midpoints (T_m). Thus, as long as the corresponding melting peaks are separated on the temperature scale, the plot of ΔH (as derived from the steepness of the transition curve) versus T_m yields slowly increasing curves leveling off at about 50°. This has been verified by calorimetric measurements [9] of polymer melting. We found similar behavior for the melting of the isolated CCA half and the recombined CCA- and pG-halves of tRNA^{Phe} (yeast) in the presence of increasing amounts of Mg^{2+} (fig. 2). Deviations from these

simple results may occur as soon as two transitions overlap on the temperature scale. Then, the apparent ΔH will grow larger and finally reach the sum of the reaction enthalpies of the individual processes, i.e. a molecule can undergo the second process only after the first. Thus, fig. 2 demonstrates the coupling between the dissociation of the recombined half molecules and the melting of the rT-stem as soon as the T_m values of these processes approach each other about 60°. Similarly, in the presence of Mg^{2+} the apparent ΔH values of peaks 1 and 2 of the whole tRNA^{Phe} (yeast) suddenly increase when these peaks overlap on the temperature scale. This occurs at about 30° in 0.01 M sodium cacodylate, pH 6.8 or at 50° in 0.01 M sodium cacodylate containing 0.1 M NaCl at pH 6.8, respectively (fig. 2). In the first case the apparent ΔH values clearly level off between 40° and 60° at the slightly increasing sum ΔH(peak 1) + ΔH(peak 2). At 60° the opening of the rT-stem is coupled to the combined peaks 1 and 2, thus producing a second steep increase in the apparent ΔH values, similar to the observations with the recombined halves. In this manner we may conclude from fig. 2 that peak 2 is coupled to peak 1 and that the melting of the rT-stem is coupled to the combined peaks 1 and 2.
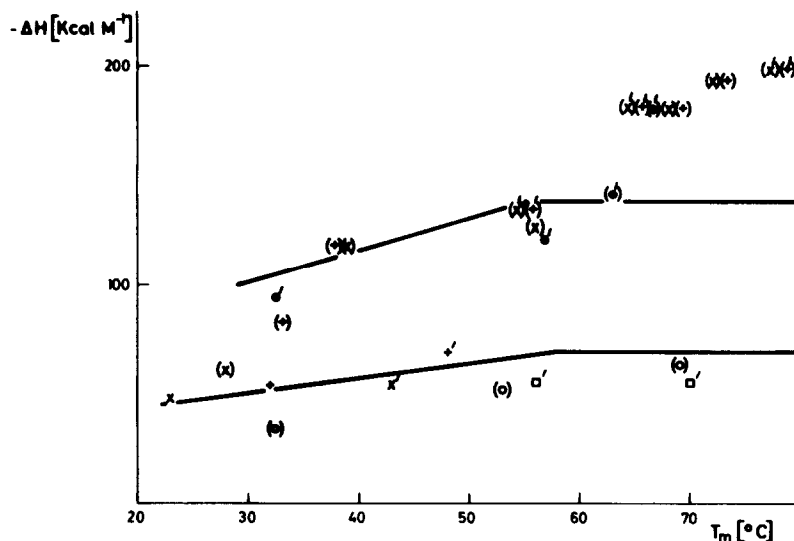
Fig. 2. Apparent reaction enthalpies ΔH as a function of T_m for several conformational transitions of tRNA^{Phe} (yeast) and its half molecules in solutions containing 0.01 M sodium cacodylate (*unprimed symbols*); 0.01 M sodium cacodylate, 0.1 M NaCl (*primed symbols*), and various concentrations of MgCl$_2$; pH 6.8. Parantheses indicate overlap of different transitions on the temperature scale, whereas values without parantheses refer to well isolated transitions. □: CCA half [5], ●: recombination of pG- and CCA halves [5]. x: peak 1, +: peak 2, o: peak 2D of the complete tRNA.
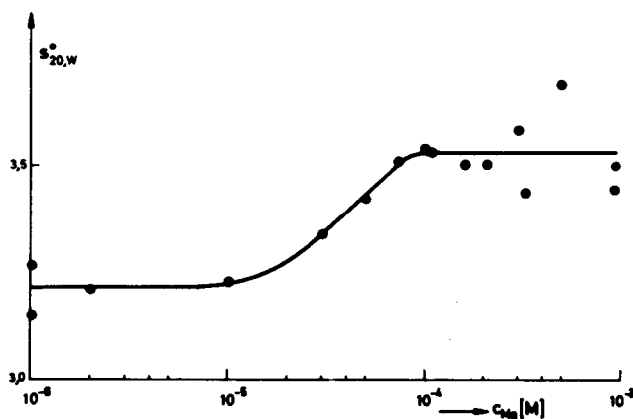

Fig. 3. Sedimentation coefficients $S^0_{20,w}$ of tRNA^{Phe} (yeast) in 0.01 M sodium cacodylate pH 6.8, containing increasing amounts of MgCl$_2$.

Further characterization of peaks 1 and 2 was achieved by ultracentrifuge and temperature jump experiments. Fig. 3 shows a pronounced increase in the sedimentation coefficient $S^0_{20,w}$ upon addition of less than 0.1 mM Mg^{2+} to a solution of tRNA^{Phe} (yeast). In the absence of Mg^{2+}, peak 1 occurs below the temperature of this experiment (20°). Whereas at 0.1 mM Mg^{2+} it is found above 20°. Except for peak 1, all of the other melting effects of tRNA^{Phe} (yeast) are affected only by higher concentrations of Mg^{2+} which do not further affect the $S^0_{20,w}$ values at 20° (fig. 3). The observed change in $S^0_{20,w}$ may thus be attributed to peak 1, which includes only 15% of the total hyperchromicity of tRNA, whereas it contributes 25% of the total change in $S^0_{20,w}$ upon heating. Hence we may conclude that peak 1 produces a relatively large change of the overall structure of tRNA^{Phe} (yeast). As judged by the single slow relaxation time of 10 msec (in addition to an unresolved fast time due to single strand unstacking) peak 1 seems to involve only two states. Peak 2, however, exhibits two slow relaxation phenomena in the msec and sec range. Further evidence for the inhomogeneity of peak 2 comes from studies
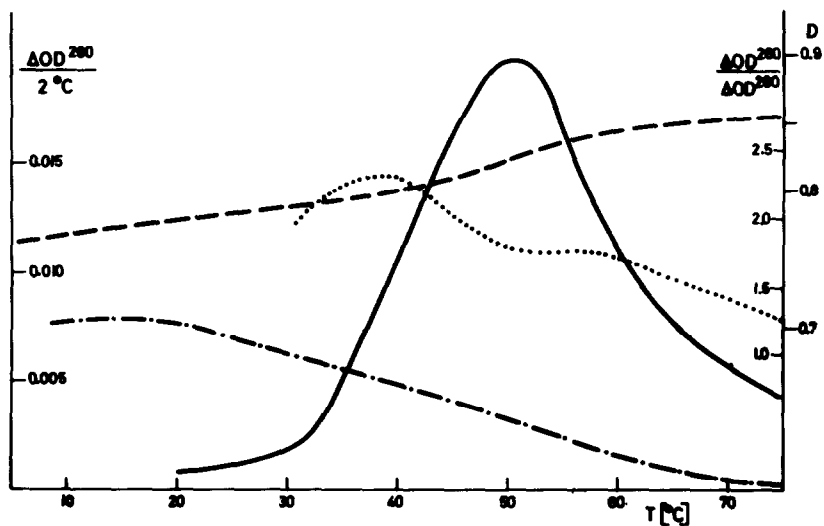
Fig. 4. Melting curves of tRNA^{Phe} (yeast) in 0.01 M sodium cacodylate 0.1 M NaCl pH 6.8.
——— : differentiated absorbance $\Delta OD^{260}/2°$;
.... : difference spectrum ratios $\Delta OD^{260}/\Delta OD^{280}$;
–·–·–· : differentiated fluorescence intensity in relative units. Excitation at 313 nm, emission at 304 nm;
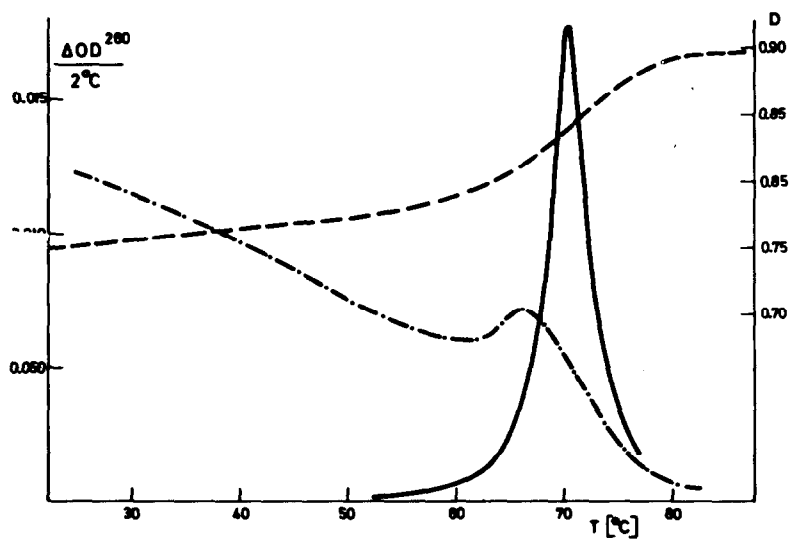- - - - : depolarization of fluorescence at the same wave lengths.

Fig. 5. Melting curves of tRNA^{Phe} in the presence of 2 mM MgCl$_2$; other conditions and symbols as in fig. 4.
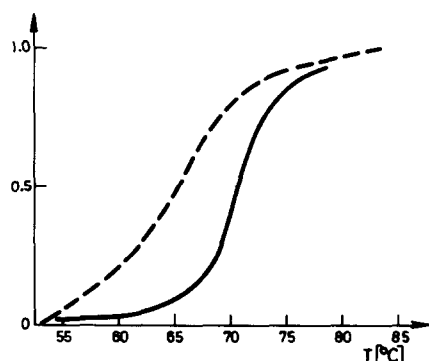
Fig. 6. Evaluation of experimental values from fig. 5:
- - - - -: degree of transition derived from fluorescence melting
curves;
———— : relative absorbance change at 260 nm.

of the fluorescence intensity and fluorescence polarization of the Y-base as a function of temperature.

Thiebe and Zachau reported fluorescence measurements with tRNA^{Phe} (yeast) [10] in the presence of Mg^{2+}; in addition to an unspecific decrease of fluorescence polarization (P) with increasing temperature, they noted a marked drop in P in the temperature range of the optical density melting. By varying experimental conditions, we have succeeded in assigning this specific decrease of P to a part of the inhomogeneous peak 2 in the absorbance-temperature profile of tRNA^{Phe} (yeast). Fig. 4 shows the melting of this molecule in the absence of Mg^{2+} as followed by absorbance, fluorescence intensity (F) and fluorescence depolarization (D). The first two curves are the derivatives with respect to temperature $(2 \times \Delta OD/\Delta T, \Delta F/\Delta T)$, whereas D is plotted directly. D and $\Delta F/\Delta T$ both exhibit unspecific changes outside the region of absorbance change upon melting. In addition, D undergoes an increase specific for peak 2 in the differential absorbance – temperature profile. Addition og Mg^{2+} reveals that this transition is not representative of complete peak 2. Fig. 5 shows melting curves analogous to the profiles of fig. 4, but in the presence of Mg^{2+}. D and $\Delta F/\Delta T$ both vary characteristically in the region of the absorbance change upon melting. The fluorescence intensity peak is somewhat shifted to lower temperatures as compared to the absorbance peak. The relative change in depolarization does not directly represent a transition curve for the concentrations of

initial and final states. In order to obtain the real degree of transition a correction for the change in fluorescence intensity has to be applied to the experimental depolarization data. The effect of this correction can easily be understood by the following qualitative argument: In the final state the fluorescence intensity is smaller than in the initial state. The depolarization of the strong fluorescence of the initial state will thus be predominant in the measured fluorescence depolarization of a mixture of molecules in the initial and final states even at equal concentrations. Hence the degree of transition apparent from the change in D will be too small. On the basis of a modified two state model (A $\rightleftharpoons$ B) for the change in D and F, a concentration transition curve $\dfrac{c(B)}{c(A) + c(B)}$ as a function of T can be derived from the experimental values of $D(T)$ and $F(T)$. A modification of the two state model allows for the unspecific change of F and D in each of the two states, i.e. fluorescence intensity and depolarization in state A and B, $F(A)$, $D(A)$, $F(B)$ may depend on temperature. For small ($<$ 10%) changes in depolarization one may easily derive the following formula for the degree of transition:

$$\frac{c(B)}{c(A) + c(B)} = \frac{D(A) - D}{D(A) - D(B)} \times \frac{1 + 2D(B)}{1 + 2D} \times \frac{F}{F(B)}$$

where the parentheses indicate to which state the concentration c, depolarization D and fluorescence intensity with unpolarized light F refer. The signs without parentheses refer to measured fluorescence depolarization and intensity. Values for $D(A)$, $D(B)$ and $F(B)$ were obtained by extrapolation from data at temperatures at which only conformation A or B is present, respectively. The relative change of $F(B)$ with temperature assumed in this extrapolation was determined under conditions, where the transition A $\rightleftharpoons$ B took place at lower temperatures (fig. 4). Such a change in conditions does not affect the relative dependence of $F(B)$ on temperature, as can be seen in the temperature range above 70°, where only conformation B is present under both conditions. Extrapolation of $D(A)$ and $D(B)$ is simpler, because both depend linearly on temperature and the slope is not affected by changing conditions such as the concentration of mono- and divalent salts. Fig. 6 gives the result of this

evaluation of the experimental data of fig. 5 together with the relative absorbance change at 260 nm. As expected from our qualitative argument, the melting transition, as measured by fluorescence depolarization is shifted to lower temperatures and is much less steep than the absorbance–temperature profile. Under these conditions peak 1, peak 2 and the melting of the rT-stem are coupled to form the very steep absorbance change. We have to conclude that the transition indicated by D is a fifth conformational change (peak 2D) of tRNA^{Phe} (yeast), largely independent of the four reactions discussed above. The latter statement is supported by a plot of ΔH versus T_m (fig. 2), which does not show any sign of coupling to peaks 1 and 2.

Kinetic experiments support this interpretation. In the temperature range, where the changes in the depolarization and absorption overlap, two well resolvable relaxation times occur, one between 1 and 2 msec and one slower than 10 msec. In the higher temperature range ($> 66°$), where most of the depolarization transition is completed, the whole denaturation process – except the very fast unstacking process – can be described with high accuracy by one single relaxation process in the time range between 10 and 3 msec depending on the temperature (conditions as in fig. 5).

4. Conclusion

By comparison with the melting behavior of the half molecules of tRNA^{Phe} (yeast) the high temperature melting of the intact molecule can be attributed to the opening of the rT- and hU-stem. In addition, three more melting processes were resolved (peaks 1, 2 and 2D). The appearance of these three transitions excludes the successive opening of the two remaining, unassigned branches of the clover leaf. Furthermore,

it can be shown that peak 2 is coupled to peak 1 and the opening of the rT-stem depends on the combined transitions 1 and 2. Peak 2D, however, is virtually independent of peaks 1 and 2. A more detailed assignment of the observed transitions to elements of secondary and tertiary structure becomes possible if one makes the following two plausible assumptions: the clover leaf branches and tertiary structure melt out as cooperative units and the opening of the anticodon stem occurs more easily when the acceptor branch has already melted because of ring closure by the latter. This leads to the assignment of peak 1 as the melting of the acceptor stem, of peak 2 as the melting of the anticodon branch, and of peak 2D as the unfolding of tertiary structure. This picture is in agreement with the difference spectrum ratios, the simple relaxation behavior in peak 1 and the more complex relaxation behavior in peak 2. In addition, this assignment reflects the similarity between the melting behavior of tRNA^{Phe} and tRNA^{Ala} from yeast. In this way, at least for these two tRNA species, a rather consistent picture seems to arise.

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