# Thermodynamic Studies of Transfer Ribonucleic Acids. II. Characterization of the Thermal Unfolding of Yeast Phenylalanine-Specific Transfer Ribonucleic Acid†

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ABSTRACT: The unfolding reaction of yeast phenylalanine-specific transfer ribonucleic acid ($tRNA^{Phe}$) was studied by equilibrium spectrophotometric and direct calorimetric measurements. The equilibrium spectrophotometric measurements demonstrated that the observed changes in optical density on thermal unfolding are independent of time and completely reversible and reproducible. Furthermore, the apparent melting temperature and the sharpness of the transition increase with increasing free $Mg^{2+}$ concentration. The isothermal calorimetric determination of the enthalpy of unfolding was carried out by initiating the reaction by removal of $Mg^{2+}$ (by complexation with ethylenediaminetetraacetic acid (EDTA)) at elevated temperature. The validity of the two-state approximation for the thermal unfolding of $tRNA^{Phe}$ is supported by data obtained from spectrophotometric, calorimetric, and preliminary kinetic experiments. The apparent enthalpy change is a monotonic function of the temperature at all $Mg^{2+}$ concentrations tested from 0 to 1 mM free $Mg^{2+}$. Furthermore, the apparent enthalpy change at 57° (127 ± 11 kcal/mole) agrees well within experimental error with the calorimetric enthalpy change determined at 57° (123 ± 25 kcal/mole). Additionally, identical thermal-unfolding behavior is observed at a variety of wavelengths, including wavelengths at which AU and GC base-pair melting can be detected independently. Finally, only a single rate-limiting process with a half-time of approximately 25 msec was observed for the unfolding of $tRNA^{Phe}$ by preliminary stopped-flow experiments where $tRNA^{Phe}$ was unfolded at 42° by removal of $Mg^{2+}$ by complexation with EDTA.

The reversible, thermal unfolding of transfer ribonucleic acids in aqueous solution can be represented as an equilibrium mixture of folded and unfolded forms of the macromolecule: folded tRNA ⇄ unfolded tRNA. In the first approximation all molecules can be considered to exist in either one of these two structural states.

Folded tRNA refers to that form of tRNA in which the predominant interactions are intramolecular—base-pair formation, base-stacking interactions, etc. In the unfolded form, on the other hand, hydrogen bonding between bases is reduced and replaced by hydrogen-bond formation with the solvent. Although this conformational isomerization reaction of tRNA has been extensively studied (Fresco *et al.*, 1966; Millar and Steiner, 1966; Adams *et al.*, 1967; Cramer *et al.*, 1968; Reeves *et al.*, 1970; Römer *et al.*, 1970a; Robison and Zimmerman, 1971) no quantitatively consistent thermodynamic picture of the reaction has yet been developed. Nonetheless, such a quantitative thermodynamic description of the transition is necessary to better understand the thermodynamic forces which determine the molecular conformation of tRNA and the influence of such factors as $Mg^{2+}$ and temperature on its biological activity.

In pursuit of the thermodynamic changes associated with tRNA unfolding, equilibrium spectrophotometric and calorimetric studies of the unfolding transition of $tRNA^{Phe}$[1] from Brewer's yeast have been made. The results of these studies indicate that the thermal-unfolding reaction can be described in terms of a relatively simple thermodynamic model. In this paper, a phenomenological characterization of the thermal-unfolding transition of $tRNA^{Phe}$ as a function of of $Mg^{2+}$ concentration and temperature will be presented. Furthermore, the validity of the two-state approximation for this reaction will be demonstrated. This result is especially significant in that it allows the thermodynamic quantities for the unfolding reaction to be obtained from equilibrium data alone. From an analysis of these thermodynamic quantities and thermodynamic information about $Mg^{2+}$ binding, a simple and self-consistent model for the thermal unfolding of $tRNA^{Phe}$ as a function of $Mg^{2+}$ concentration and temperature has been developed. This model and some of its biological implications will be presented in the following paper.

## Experimental Section

*Materials.* The $tRNA^{Phe}$ (lots 6269501, 6449304, 7130103, and 7150206) was purchased from Boehringer-Mannheim, where it was purified from Brewer's yeast according to the method of Wimmer *et al.* (1968) with the exception that the last step, chromatography on silicic acid coated benzylated DEAE-cellulose, was omitted (Bergmeyer, H. U., and Weimann, G., personal communication). The activity of $tRNA^{Phe}$ was about 1000 μμmoles of phenylalanine accepted/$A_{260}$ unit which represented 96–99% of the total biological activity

† From the Department of Physiological Chemistry, The Johns Hopkins School of Medicine, Baltimore, Maryland. 21205. *Received June 19, 1972.* This investigation was supported by grants from the National Science Foundation (GB-7243) and National Institutes of Health (CA-11693).

‡ Predoctoral Trainee supported by National Institutes of Health Grant GM-00181, 1967–1971.

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[1] Abbreviations used are: $tRNA^{Phe}$, yeast phenylalanine-specific transfer ribonucleic acid; $Na_2EDTA$ disodium ethylenediaminetetraacetate.

of tRNA in the different lots. The ethylenediaminetetraacetic acid (EDTA) used was Fisher ACS certified grade. All other reagents were of the highest purity commerically available.

The lyophilized $tRNA^{Phe}$ was stored at 4° in the dark until used. Solutions of $tRNA^{Phe}$ were prepared by dissolving it (without further purification) in buffer containing 5 mM $Na_2HPO_4$–$NaH_2PO_4$, 5 mM NaCl, and 1.5–5 mM EDTA at pH 7.2 at a concentration of 0.5–1.0 mg of $tRNA^{Phe}$/ml of buffer. This solution was placed in dialyzer tubing that had been boiled twice for 30 min in 0.2 M EDTA–0.1 M $K_2CO_4$ (pH 10) and then rinsed extensively with distilled water. The solution was then dialyzed for 2 hr at 4° against at least 100 volumes of buffer containing 5 mM $Na_2HPO_4$–$NaH_2PO_4$, 5 mM NaCl, and 1.5–5 mM EDTA at pH 7.2 to insure complete complexation by EDTA of the $Mg^{2+}$ in the $tRNA^{Phe}$ sample. Further dialysis of the sample against 2000 volumes of buffer containing 5 mM $Na_2HPO_4$–$NaH_2PO_4$ and 5 mM NaCl for approximately 24 hr at 4° removed the Mg–EDTA complex. The absence of $Mg^{2+}$ was verified by the fact that the heat of mixing of this solution with excess EDTA produced no calorimetrically measurable heat except for that of dilution of the various components. This means that less than 0.3 mole of $Mg^{2+}$ $tRNA^{Phe}$ was present in our solutions (Rialdi *et al.*, 1972). The sample was then dialyzed against 2000 volumes of the same buffer containing a known amount of $MgCl_2$ for about 24 hr in the cold.

Dialyzed $tRNA^{Phe}$ solutions to be used in spectrophotometric experiments were further diluted approximately 1:250 or 1:500 with the last dialysis buffer and used within 10 days. The concentration of $tRNA^{Phe}$ was determined by absorbance measurements at 257 mμ using an extinction coefficient of 540,000 l./(mole cm) (Levy, 1971). All solutions were degassed for 30 min, and heated to 60° for 10 min, and cooled to 20° before use. The preparation of $tRNA^{Phe}$ solutions used for the calorimetric experiments has been previously described (Rialdi *et al.*, 1972).

*Thermal-Unfolding Experiments.* The thermal-unfolding experiments were performed in a Cary 14 spectrophotometer. The sample compartment was equipped with an especially designed Plexiglass cuvet holder for a Pyrocell cylindrical water-jacketed cuvet with 1-cm path length. The reference solution was placed in a standard cuvet in a thermostatic block in the reference compartment. The temperature of the solutions was measured with a precision of ±0.02° with a Cole-Parmer digital read-out thermistor probe immersed directly in the solutions. The temperature of the reference solution was maintained at 20°. The sample temperature was varied in either direction in a novel way with a Pohl-type (Pohl, 1968) temperature-jump apparatus constructed of three three-way solenoid valves, which quickly switched the fluid circulating around the cuvet between two thermostatic water baths. Changes in the absorbance difference between sample and reference solutions were recorded on an expanded (0–0.1 *A* unit) scale with an accuracy of 0.001 unit. The performance of this system is discussed in the Results section.

Calorimetric measurements were made with an LKB 10700-2 differential microcalorimeter, the useful sensitivity of which is on the order of 0.1 μcal/sec. The accuracy of this calorimeter was checked by measurement of the heat of dilution of sucrose and heat of neutralization of HCl with excess NaOH (Zimmer and Biltonen, 1972). The unfolding transition was initiated in the calorimeter by mixing $tRNA^{Phe}$ in 1 mM $MgCl_2$ with excess EDTA at 57°.

The unfolding transition was initiated in the stopped-flow apparatus, which consisted of an Aminco-Morrow stopped-flow device adapted to a Beckman DU spectrophotometer, by mixing $tRNA^{Phe}$ in 1 mM free $Mg^{2+}$ with excess EDTA at 42°. The signal was amplified and recorded on a Honeywell Visicord recorder. The performance of the instrument was checked for the reaction of HCl and bicarbonate using Bromophenol Blue as an indicator.

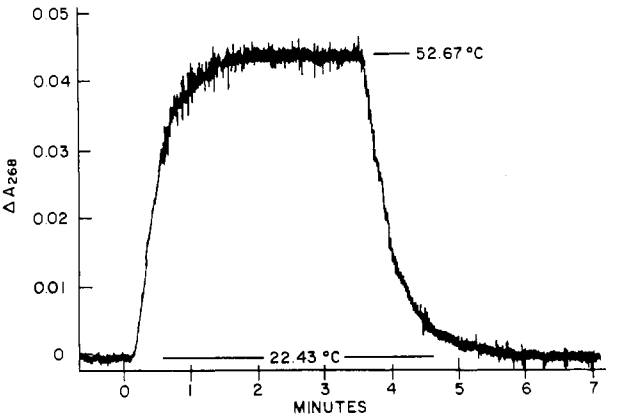


FIGURE 1: A typical temperature-jump cycle for the unfolding of $tRNA^{Phe}$. Solution conditions were: 0.4 μM $tRNA^{Phe}$, 5 mM $NAHPO_4$–$NaH_2PO_4$, pH 7.1.

## Results and Analysis

*Thermal-Unfolding Experiments.* It has previously been shown that the thermal-unfolding transition of tRNA is accompanied by a large hyperchromic change in its ultraviolet absorption spectrum (Fresco *et al.*, 1963). Thus temperature-dependent absorption changes provide a convenient method by which to monitor the thermal unfolding of tRNA. However, in order to obtain meaningful equilibrium data it is necessary to first establish that the observed changes in absorbance are independent of time and completely reversible. Initial studies of the thermal unfolding of $tRNA^{Phe}$ indicated that neither of these criteria were easily met. The primary problem was found to be the result of a slow, but appreciable aggregation of the unfolded form of the molecule at high temperature. This fact was established by a comparison of the magnitude of the increased light scattering with the magnitude of the slow irreversible changes observed in the absorption spectrum when the sample was maintained at elevated temperatures for an extended period of time. However, when the temperature of the sample was quickly raised (about 1 min), the absorbance change was independent of time for several minutes; and when the temperature was lowered, complete reversibility was achieved. This rapid change in temperature was achieved using a Pohl-type (Pohl, 1968) temperature-jump apparatus in which the source of the circulating fluid in the cell was quickly changed between two circulating water baths maintained at different temperatures.

A typical temperature cycle is shown in Figure 1. In this particular experiment the temperature was "jumped" by approximately 30° to extensively unfold the $tRNA^{Phe}$ and after about 1.5 min both temperature and the absorbance difference were constant for several minutes. Upon lowering the temperature complete reversibility of the absorbance change was observed. For subsequent data points the sample was maintained at this low temperature until the temperature of the other bath could be changed. In this way the sample was subjected to high temperature for only a short period of the total time of the experiment.

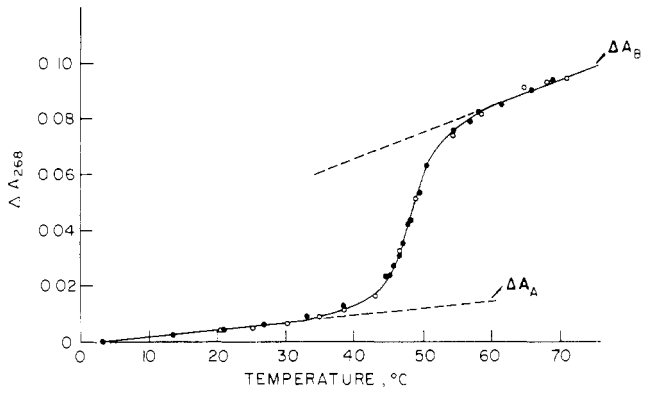


FIGURE 2: A typical thermal-unfolding profile for $tRNA^{Phe}$. Solution conditions: 0.7 $\mu$M $tRNA^{Phe}$, 5 mM $NAHPO_4$–$NAH_2PO_4$, 5 mM NaCl, pH 7.2. Although no $Mg^{2+}$ was added to the solution, this particular sample was not $Mg^{2+}$ free. The open and filled circles are data obtained with two different aliquots of the same sample. The broken lines labeled $\Delta A_A$ and $\Delta A_B$ represent the temperature dependence of the absorbance of $tRNA^{Phe}$ in states A and B, respectively.

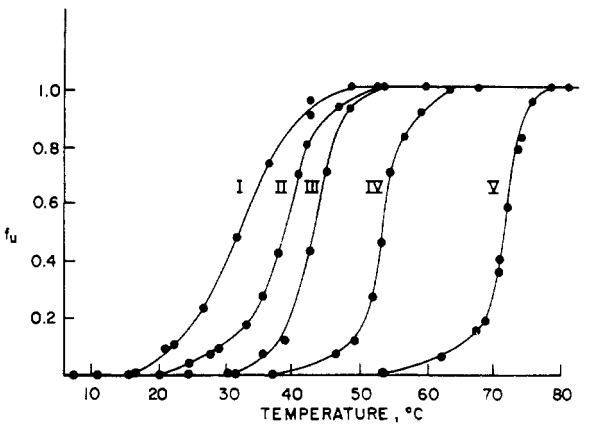


FIGURE 3: Representative melting profiles of $tRNA^{Phe}$ in 5 mM $NaHPO_4$–$NaH_2PO_4$ under varying conditions of NaCl and $MgCl_2$. $f_u$ was calculated as described in the text. I, no NaCl 50 $\mu$M $Na_2$-EDTA; II, 5 mM NaCl–no $MgCl_2$; III, 12.5 mM NaCl no $MgCl_2$; IV, 5 mM NaCl–50 $\mu$M $MgCl_2$; V, 5 mM NaCl–1 mM $MgCl_2$.

The reproducibility of the data for a particular unfolding transition is demonstrated in Figure 2. The closed circles are data obtained with one aliquot of a sample in which the temperature jumps were made in a semirandom fashion, *i.e.*, absorbance changes observed at some intermediate temperatures were measured after the sample had previously been more extensively unfolded by temperature jumps to higher temperatures. These data show the absence of any hysteresis effects on the transition curve. The open circles were data obtained with another aliquot of the same sample stored for approximately 10 days in the cold. The data obtained in the two sets of experiments are indistinguishable and clearly satisfy our criteria for reversibility and reproducibility. Similar data have been obtained over a wide range of conditions.

*Temperature Data and Thermodynamic Calculations.* Data such as that presented in Figure 2 were used to calculate $f_u$, the degree of unfolding of $tRNA^{Phe}$, as a function of temperature and solvent composition in the following way. The two extrapolated lines, labeled $\Delta A_A$ and $\Delta A_B$, were assumed to represent temperature-dependent changes in the absorbance of the pure folded state A, and the pure unfolded state B.[2] $f_u$ is then defined by eq 1. The results of such treatment of the

$$f_u = (\Delta A - \Delta A_A)/(\Delta A_B - \Delta A_A) \quad (1)$$

data are shown in Figure 3 for several representative $tRNA^{Phe}$ transition curves. From these results it is evident that the thermal-unfolding behavior of $tRNA^{Phe}$ is very dependent on the solution conditions, especially the free $Mg^{2+}$ concentration. Both the melting temperature, $T_m$, which is defined as that temperature at which $f_u = 0.5$, and the sharpness of the transition curve increase with increasing free $Na^+$ and $Mg^{2+}$ concentration.

[2] The assumed linear temperature behavior of $\Delta A_A$ and $\Delta A_B$ is only a first approximation. Errors in this extrapolation will, of course, produce errors in the calculated values of $f_u$ and in any calculated thermodynamic quantities. However, the temperature dependencies of $\Delta A_A$ and $\Delta A_B$ outside the transition region appear to be accurately represented as linear functions. Because of this, and because the extrapolations are not large (generally less than 15°), we estimate that errors in $f_u$ due to errors in the extrapolations are certainly less than 10%.

Apparent thermodynamic quantities for the transition under a variety of conditions can be calculated as a function of temperature, assuming that the thermal unfolding of $tRNA^{Phe}$ is a two-state transition. If this assumption is true, then $f_u$ is equal to the fraction of molecules in state B ($1 - f_u$), the fraction in state A, and the apparent equilibrium constant, $K_{app} = f_u/(1 - f_u)$. The standard free-energy change is given by

$$\Delta G^\circ = -RT \ln K_{app} \quad (2)$$

The standard enthalpy change and entropy change can be calculated at any temperature from

$$\Delta H^\circ = -R[\partial \ln K_{app}/\partial(1/T)]_p \quad (3)$$

and

$$\Delta S^\circ = -\left[\frac{\partial \Delta G^\circ}{\partial T}\right]_p \quad (4)$$

The van't Hoff enthalpy change was determined from eq 3 at the $T_m$ for several transition curves obtained at different $Mg^{2+}$ concentrations. These heats were found to be linearly related to the $T_m$ as shown in Figure 4. Consequently the van't Hoff heat at any temperature can be estimated by interpolation using the solid line (Figure 4) obtained by a linear least-squares analysis of the results and given by the equation, $\Delta H = 3.0T - 44.3$, where $T$ is the temperature in degrees Celsius.

*Calorimetric Experiments.* From the results in Figure 3 it is apparent that at certain temperatures, $tRNA^{Phe}$ exists in the unfolded state, B, in the absence of $Mg^{2+}$, and in the folded state, A, when the free $Mg^{2+}$ concentration is 1 mM. It is thus possible to isothermally unfold $tRNA^{Phe}$ at elevated temperatures (*e.g.*, 57°) by removal of $Mg^{2+}$ by complexation with excess EDTA. Thus, when $tRNA^{Phe}$ equilibrated with 1 mM $MgCl_2$ is mixed with excess EDTA in the flow microcalorimeter, the following reaction occurs

$$\begin{array}{ccccc} A\,(Mg^{2+})\ r_A & \rightleftharpoons & Mg^{2+}\ (free) & \xrightarrow[Q]{EDTA} & B_0 + EDTA\sim Mg^{2+} \\ \downarrow Q_1 & & & & \uparrow Q_3 \\ A_0 + Mg^{2+}\ (released) & + & Mg^{2+}\ (free) & \xrightarrow[EDTA]{Q_2} & A_0 + EDTA\sim Mg^{2+} \end{array}$$

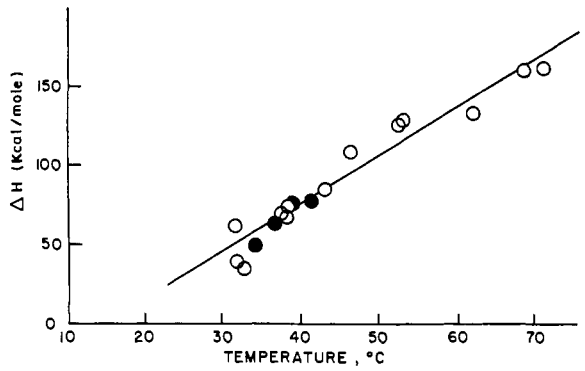


FIGURE 4: The van't Hoff heat, calculated as described in the text, as a function of temperature. The open circles are values calculated at the $T_m$ for experiments performed at varying $Mg^{2+}$ concentration. The filled circles represent values of $\Delta H^\circ$ at different temperatures as obtained from experiments with no $Mg^{2+}$. In all cases the NaCl concentration was 5 mM. The solid line is calculated according to the equation $\Delta H = 3.0T - 44.3$.

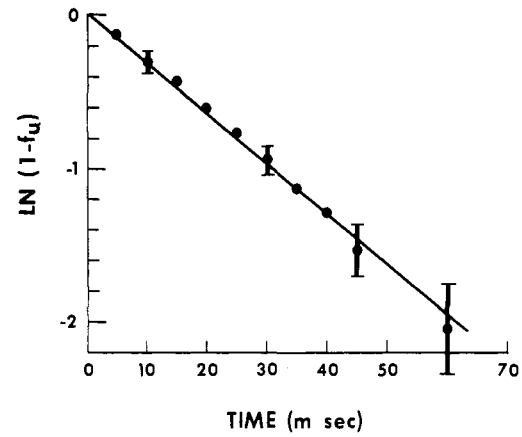


FIGURE 5: Ln $(1 - f_u)$ *vs.* time. The fraction of $tRNA^{Phe}$ molecules unfolded, $f_u$, was calculated from time-dependent changes in the transmittance at 268 nm as described in the text.

where A and B designate the folded and unfolded forms of the macromolecule, respectively. The steady-state heat generated ($Q_s$) divided by the flow rate ($F$), is

$$Q = \frac{Q_s(\text{cal/sec})}{F(1/\text{sec})} = Q_{dil} + Q_1 + Q_2 + Q_3 \qquad (5)$$

$Q_{dil}$ is the sum of the heats of dilution of the various components and was measured separately.

The heat associated with the release of $r_A$ molecules of $Mg^{2+}$ from $tRNA^{Phe}$ in state A is

$$Q_1 = -r_A C_t \Delta H_A \qquad (6)$$

where $r_A$ is the average number of $Mg^{2+}$ bound per $tRNA^{Phe}$ and has been determined to be 22 ± 1 at 1 mM $Mg^{2+}$ (Rialdi *et al.*, 1972). $C_t$, the concentration of $tRNA^{Phe}$, was determined spectrophotometrically. $\Delta H_A$, the molar heat of binding of $Mg^{2+}$ to the macromolecule, has previously been shown to be zero (Rialdi *et al.*, 1972) so that $Q_1 = 0$. The heat associated with complex formation between $Mg^{2+}$ and EDTA is

$$Q_2 = (M + r_A C_t)\Delta H_E \qquad (7)$$

where $M$ is the initial concentration of free $Mg^{2+}$. $\Delta H_E$, the molar heat of reaction between $Mg^{2+}$ and EDTA, was determined by measuring that heat of reaction between standard EDTA and $MgCl_2$ solutions. $\Delta H_E$ was directly determined by measuring the heat of reaction between EDTA and the last buffer solution against which $tRNA^{Phe}$ was dialyzed.[3] Since $r_A$, $C_t$, and $\Delta H_E$ are known, $Q_2$ can now be calculated.

The heat associated with reaction 3, the $A_0 \rightarrow B_0$ transition in the absence of free $Mg^{2+}$, is

$$Q_3 = C_t \Delta H_t \qquad (8)$$

where $\Delta H_t$ is the molar heat of $tRNA^{Phe}$ unfolding in the absence of free $Mg^{2+}$ at 57°. Thus $\Delta H_t$ can be readily calculated from the following relationship

$$\Delta H_t = \frac{Q_3}{C_t} = \frac{Q - Q_{dil} - Q_1 - Q_2}{C_t} \qquad (9)$$

$\Delta H_t$ was found to be 123 ± 25 kcal/mole at 57°.

*Kinetic Experiments.* Preliminary experiments on the kinetics of unfolding of $tRNA^{Phe}$ were carried out in a manner exactly analogous to the calorimetric experiments. $tRNA^{Phe}$, dialyzed against 1 mM $MgCl_2$, was mixed with excess EDTA in the stopped-flow apparatus at 42°. A single rate-limiting process, as followed by a decrease in transmittance, was observed. The extent of reaction as a function of time was calculated by

$$f_u = \frac{\ln (t_0/t)}{\ln (t_0/t_f)} \qquad (10)$$

where $t$ is the transmittance of the $tRNA^{Phe}$ solution at any time and $t_0$ and $t_f$ are the initial and final transmittance values. A plot of ln $(1 - f_u)$ *vs.* time yielded a straight line, as shown in Figure 5, and an estimate of $k_d$, the rate constant for unfolding, of 50 $sec^{-1}$ was obtained.

*Validity of the Two-State Approximation.* The results presented in Figure 3 clearly show that the thermal-unfolding behavior of $tRNA^{Phe}$, as characterized by the $T_m$ and the sharpness of the transition curves, is very dependent on the temperature and free $Mg^{2+}$ concentration. In order to dissect the influence of each factor, it is necessary to know the apparent thermodynamic quantities for the unfolding of $tRNA^{Phe}$ as a function of temperature and $Mg^{2+}$ concentration. In the present case, such information can be obtained from the results in Figure 3 if, and only if, the transition can be well approximated as a two-state transition (Lumry *et al.*, 1966; Tanford, 1968). Such a transition is one in which, under equilibrium conditions, a mixture of two states, or two thermodynamic distributions, can account for essentially all the molecules; any intermediate states which may exist are negligibly populated. Several tests have been developed to determine the validity of this approximation for macromolecular conformational transitions in general, and can be applied to the present data for the thermal unfolding of $tRNA^{Phe}$.

[3] Due to the Donnan membrane effect, the concentration of free $Mg^{2+}$ in the $tRNA^{Phe}$ solution is not identical with that in the last dialysate. This difference corresponds to approximately 1 $Mg^{2+}$ per $tRNA^{Phe}$ molecule or about 9 kcal/mole of $tRNA^{Phe}$. This correction is small compared to the error in $Q$ and was not taken into account.

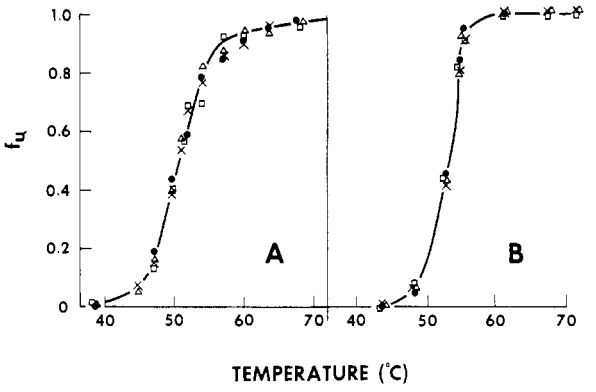


FIGURE 6: A plot of $f_u$ *vs.* temperature using different wavelengths to monitor the transition. ○, 280 nm; ×, 268 nm; △, 260 nm; ●, 240 nm. Curve A was obtained under the solvent conditions as described in Figure 2. Curve B was obtained in 5 mM $Na_2HPO_4$–$NaH_2PO_4$, 5 mM NaCl, 50 μM $MgCl_2$.

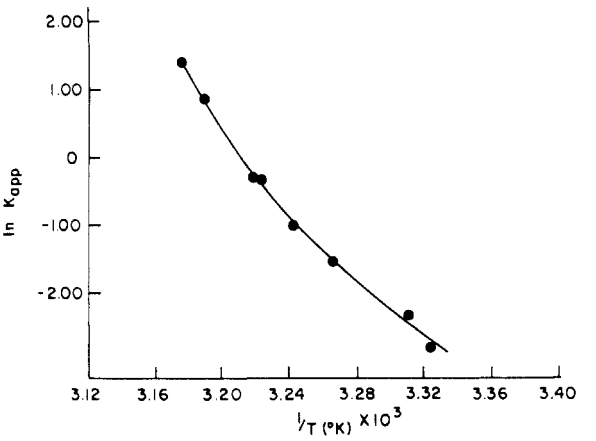


FIGURE 7: Van't Hoff plot of the data shown in curve II, Figure 3, assuming a two-state transition.

COMPARISON OF OBSERVABLES. For two-state transitions, the form of the transition curve (*i.e.*, the temperature-dependence of $f_u$) must be independent of the physical observable used to monitor the transition (Lumry *et al.*, 1966). In the case of nucleic acids, for example, if GC-rich regions melt independently of AU-rich regions, the transition profile obtained by absorbance difference measurements will be wavelength dependent since AU and GC base-pair melting can be detected independently at different wavelengths (Felsenfeld and Sandeen, 1962). If the transition is two state and all AU and GC base-pairs unfold simultaneously, the profile will be independent of the wavelength used to monitor the reaction. In Figure 6, the fraction of molecules unfolded as a function of temperature, as calculated from absorbance difference measurements at several wavelengths, is shown for two different sample preparations. Support for the validity of the two-state approximation in both cases is provided by the fact that no deviation outside of experimental error is observed for the melting profiles obtained at different wavelengths.

DEPENDENCE OF THE APPARENT ENTHALPY CHANGE ON TEMPERATURE. For a transition exhibiting two-state behavior, the enthalpy change is constant or varies monotonically with the temperature (Lumry *et al.*, 1966; Poland and Scheraga, 1965). Any temperature dependence of the enthalpy change must be the result of a heat capacity difference between forms of the molecule. For a multistate transition, the enthalpy change will exhibit an extremum near the transition temperature. In Figure 7, the van't Hoff plot for curve II in Figure 3 is shown. No inflection point is observed although the slope does increase with temperature. Similar results have been obtained from van't Hoff plots for unfolding under a variety of conditions. Thus these data support the validity of the two-state approximation for the $tRNA^{Phe}$ unfolding reaction and suggests a heat capacity difference between forms.

COMPARISON OF THE CALORIMETRIC AND VAN'T HOFF ENTHALPY CHANGES. It has previously been shown that the calorimetric heat is always greater than the van't Hoff heat for a multistate conformational transition (Lumry *et al.*, 1966). Only in the case of a two-state transition is equality between these two enthalpy changes observed. Our calorimetric experiments show the enthalpy change for unfolding $tRNA^{Phe}$ at 57° is 123 ± 25 kcal/mole. The van't Hoff values was found by interpolation of the results in Figure 4 to be 127 ± 11 kcal/mole at the same temperature. This agrees well within the respective errors to the calorimetric enthalpy change. This favorable comparison between the van't Hoff heat and calorimetrically determined enthalpy change provides further strong support for the validity of the two-state approximation for the thermal unfolding of $tRNA^{Phe}$.

KINETICS OF UNFOLDING. For a two-state transition, a plot of the ln of the fraction of molecules folded *vs.* time should be linear (Tanford, 1968). For the unfolding of $tRNA^{Phe}$ a single relaxation process with a half-time of approximately 25 msec was observed and a plot of the ln $(1 - f_u)$ *vs.* time was linear within experimental error. These results indicate that there is a single rate-limiting step in $tRNA^{Phe}$ unfolding which displays first-order kinetics. They do not, however, rule out the existence of other faster reactions being involved in a consecutive reaction scheme. Nevertheless, these kinetic results are consistent with a two-state transition and provide additional support for the validity of the two-state approximation of $tRNA^{Phe}$ thermal-unfolding reaction.

## Discussion

The meaning and significance of the two-state approximation for macromolecular conformational changes have previously been thoroughly discussed (Lumry *et al.*, 1966; Tanford, 1968; Jackson and Brandts, 1970). The tests which are used to determine the validity of this approximation for a particular transition have been applied to several protein systems (Brandts and Hunt, 1967; Biltonen and Lumry, 1969; Jackson and Brandts, 1970; Hermans and Rialdi, 1965; Atha and Ackers, 1971; Tanford, 1968). This study represents the first application of these tests to a tRNA system. The calorimetric, spectrophotometric, and kinetic data reported herein clearly support the validity of the two-state approximation for the $tRNA^{Phe}$ thermal unfolding.

The validity of the two-state approximation as applied to the thermal unfolding of $tRNA^{Phe}$ means that there are only two distinguishable macroscopic distributions of conformational states available to $tRNA^{Phe}$ molecules under equilibrium conditions; the population of any intermediate states is negligible. The appellation "two-state transition" is only a thermodynamic description of the transition. It does not provide any description of the states nor any mechanistic details of the transition between the two thermodynamic states. Two-state transitions are unique, however, in that in contrast to multistate transitions, the true thermodynamic

parameters for the reaction can be determined from equilibrium measurements alone without any further assumptions.

In the case of $tRNA^{Phe}$, it is apparent from accumulated experimental evidence (Fresco *et al.*, 1963; Fresco, 1963, Beardsly and Cantor, 1970) that the low temperature state is comprised of folded $tRNA^{Phe}$ molecules which are extensively base paired intramolecularly and that the high-temperature state is comprised of non-base-paired molecules that are probably hydrogen bonded to water. It is impossible to define either of these states in detailed atomic terms because they actually represent the average of a distribution of molecular species. For example, unfolded $tRNA^{Phe}$ includes all molecules with no intramolecular hydrogen bonds, but these molecules vary in their degree of base stacking from 0 to 100%. The average structure varies relatively noncooperatively with temperature (Poland *et al.*, 1966) as reflected in the temperature dependence of certain physical properties, such as the absorbance of the folded and unfolded state (Figure 2), and in the temperature dependence of the enthalpy of transition, which is at least partially due to an increased heat capacity in the unfolded state (Klump *et al.*, 1960; Rawitscher *et al.*, 1963; Crothers, 1971).

The first two tests for two-state behavior have no direct bearing on the question of whether $Mg^{2+}$ binding to folded $tRNA^{Phe}$ induces a structural change in the macromolecule; their results simply imply that the unfolding transition is approximately two state under a given set of solvent conditions. The kinetic experiment failed to detect a structural change upon $Mg^{2+}$ removal, prior to the unfolding reaction. However, this result is inconclusive.

The agreement obtained between the van't Hoff and calorimetric enthalpy changes for unfolding has several ramifications. First, it is the clearest evidence that good estimates of the thermodynamic quantities for unfolding of $tRNA^{Phe}$ can be obtained from equilibrium data assuming two-state behavior. Secondly, since the calorimetric enthalpy change applies to unfolding at 57° in the absence of $Mg^{2+}$ whereas the van't Hoff heat corresponds to unfolding at 57° in approximately 60 $\mu$M $Mg^{2+}$, this agreement suggests that $Mg^{2+}$ does not significantly influence the enthalpy change of the reaction. This statement is consistent with the observation that the enthalpy of $Mg^{2+}$ binding to $tRNA^{Phe}$ is $0 \pm 100$ cal/mole of $Mg^{2+}$ bound and further implies that $Mg^{2+}$ does not effect, within current experimental error, the average enthalpy of the molecular distribution of either the folded or unfolded state. It should be noted that $Mg^{2+}$-induced changes in the fluorescence of the Y base of $tRNA^{Phe}$ suggest that some type of structural alteration occurs upon $Mg^{2+}$ binding in the folded state (Eisinger *et al.*, 1970; Romer *et al.*, 1970). This does not appear to be accompanied by any observable change in the ultraviolet absorption spectrum ($\lambda \sim 260$ nm) of $tRNA^{Phe}$, however (Levy, 1971). In addition Rialdi *et al.* (1972) were unable to detect any enthalpy change ($\pm 2$ kcal/mole) upon $Mg^{2+}$ binding which could be attributed to a conformational change in the macromolecule. It thus is unlikely that $Mg^{2+}$ binding produces any *major* conformational change in either the folded or unfolded form of $tRNA^{Phe}$, and any conformational change induced by $Mg^{2+}$ is probably very local in nature. This conclusion is important in the development of the thermodynamic model of $tRNA^{Phe}$ unfolding which will be presented in the following paper (Levy and Biltonen, 1972).

The tests which have been used to verify the validity of the two-state approximation of $tRNA^{Phe}$ unfolding are actually tests to establish the existence of multistate behavior. The absence of any evidence for such behavior is taken as support for the two-state approximation. Therefore, because of the nature of these tests, no one test provides rigorous proof for two-state behavior. For example, although the kinetic observation of one rate-limiting step is consistent with a two-state transition, it does not exclude the existence of more rapid steps in the reaction scheme. Also, the fact that the absorbance change monitored at several wavelengths yields identical unfolding behavior simply means that only two spectroscopically distinct species exist in solution. However, there may be additional species which can be detected only by techniques which monitor other physical characteristics or which are more precise.

The two-state hypothesis is an approximation to the real situation, and the validity of the approximation is only a statement of the available experimental facts. It is quite possible that as data of increased precision or data from other techniques becomes available, the existence of thermodynamically significant intermediate states may become evident. However, within the current experimental error of 5–10%, the thermal unfolding of $tRNA^{Phe}$ can be well approximated as a two-state transition.[4] Consequently, the thermodynamic quantities for the reaction can be obtained from equilibrium data alone.

Although we have established the validity of the two-state approximation for yeast $tRNA^{Phe}$, this does not necessarily imply that all tRNA molecules will exhibit two-state behavior. The tests for validity of this approximation must be applied to each tRNA species individually. It should be noted that the purity of a particular tRNA being tested is an especially important factor in determining two-state behavior. It has been well established that all tRNA molecules are *not* thermodynamically identical (Reeves *et al.*, 1970; Cramer *et al.*, 1968; Fresco, 1963; Römer *et al.*, 1970b). Reeves *et al.* (1970) have demonstrated that two tRNA molecules, which are specific for the same amino acid, in this case alanine, but which differ in sequence, have different thermal-unfolding characteristics. Much of the early published data on tRNA was done with bulk or mixed tRNA. Since each species present has its own melting characteristics, apparent multistate behavior will generally be observed for mixed samples. Furthermore, even studies on "purified" amino acid specific tRNA may not be reliable. For example, Riesner *et al.* (1969) observed multistate melting behavior with yeast $tRNA^{Ala}$ but, as mentioned, yeast $tRNA^{Ala}$ contains at least two different species which have different thermodynamic properties (Reeves *et al.*, 1970).

In summary, the thermal unfolding of $tRNA^{Phe}$ can be well approximated as a two-state transition. The equilibrium between the folded and unfolded forms is strongly influenced by the free $Mg^{2+}$ concentration. The $T_m$ and sharpness of the

[4] Römer *et al.* (1970a) have reported the "resolution of five conformational transitions" for $tRNA^{Phe}$, a conclusion which appears to be in direct contradiction to our present conclusion. It is impossible, however, to quantitatively evaluate their analysis since their data are limited and their analysis qualitative. It appears to us that a portion of the apparent discrepancy is the result of differences in the definition of a "conformational transition." For example, we regard the temperature-dependent variation in the degree of base stacking in the unfolded form, not as a distinct conformational transition, but rather as a change in the average species of the distribution of unfolded molecules, whereas Römer *et al.* (1970a) consider this to be two overlapping transitions (opening of the rT and dihydrouridine stems). Insofar as we have made a quantitative interpretation of extensive experimental data and have developed a mathematically simpler explanation, we can find little basis for the more complicated interpretation of Römer *et al.* (1970a).

transition increase with $Mg^{2+}$ concentration indicating that $Mg^{2+}$ stabilizes the folded form. Thermodynamic parameters for the transition can be obtained from equilibrium data alone since the two-state approximation is valid. From an analysis of the combined thermodynamic parameters for $tRNA^{Phe}$ unfolding and for $Mg^{2+}$ binding to $tRNA^{Phe}$ (Rialdi *et al.*, 1972), a simple, consistent model for the thermal-unfolding behavior of $tRNA^{Phe}$ as a function of $Mg^{2+}$ concentration and temperature has been developed and will be presented in the following paper (Levy and Biltonen, 1972). It will be shown that the stabilization of the folded form of $tRNA^{Phe}$ by $Mg^{2+}$ is most likely the result of $Mg^{2+}$ binding better to the folded form than the unfolded form, thereby increasing the apparent free-energy change for the reaction. Furthermore, it will be shown that the $\Delta H$ for the unfolding of $tRNA^{Phe}$ is primarily a function of temperature. This temperature dependence of the $\Delta H$ is probably the result of an increase in the heat capacity in the unfolded form.

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