Assignment 4 - Answers

Thermodynamic Analysis of RNAseA Denaturation by UV-Vis Difference Absorption Spectroscopy (and Differential Scanning Calorimetry).

The accompanying excel file (Origin_Assign_4_Data.xlsx) contains two sets of data. The first set of data represents the change in molar absorptivity at 280 nm ($\Delta\varepsilon_{280}$) versus temperature of ribonuclease A (RNAseA). The second data set are the results of a DSC experiment in which the heat capacity (C_P) was measured as a function of temperature.

1. Use the Data for RNAse to estimate:

- ***a.*** *T_M, the transition temperature (where K = [D]/[N] = 1),*
- ***b.*** *ΔH and ΔS of denaturation (at the transition temperature), and*
- ***c.*** *ΔC_P of denaturation.*
- ***d.*** *Provide a write-up that outlines the procedure you used to estimate these parameters. Include (d1) your rationale and procedure for calculating baselines, (d2) the method for determining K as a function of temperature, (d3) a plot of ΔG as a function of temperature, and (d4) the procedure for determining ΔH and ΔS of denaturation (at the transition temperature).*
- ***e.*** *Calculate the temperature of maximum stability.*
- ***f.*** *Generate a protein stability plot (ΔG versus T) based on the fit parameters generated in part **d** and use the plot to predict the T_M of cold denaturation.*

1. Thermal Denaturation of Ribonuclease A by UV-Vis Difference Absorption Spectroscopy.

The difference in the molar absorbancy at 280 nm ($\Delta\varepsilon_{280}$) of a single domain protein, Ribonuclease A, was recorded in water as a function of temperature and is summarized in the Table below.

Temperature, K	$-\Delta\varepsilon_{280}$
281.74	0
290.76	0.008
294.62	0.009
297.57	0.018
300.27	0.037
302.72	0.044
304.87	0.059
307.08	0.082
309.78	0.133
311.99	0.215
314.13	0.363
316.1	0.593
318.3	0.911
320.02	1.17
322.23	1.355
324.62	1.444
326.58	1.467
330.27	1.459
333.64	1.437
335.85	1.415
339.53	1.385
343.15	1.36

Question 1 Answers

Parts a-d. The difference in the absorbance of Ribonuclease A ($\Delta\varepsilon_{280}$) is analyzed following an approach in which (1) the calculated linear fits to the low and high temperature baselines are extrapolated through the transition region, (2) the equilibrium constants are calculated as a function of temperature, (3) the free energies are obtained from the equilibrium constants, and (4) the free energy is fit to a polynomial function to estimate T_M and the thermodynamic parameters associated with denaturation.

The table shows the values of the baselines for the native and denatured forms ($-\Delta\varepsilon_N$ and $-\Delta\varepsilon_D$, respectively), which were determined by extrapolation of the linear regression preformed on the first and last four data points of $-\Delta\varepsilon_{280}$ (respectively). Values of K are then calculated using the formula $(-\Delta\varepsilon_{280} - (-\Delta\varepsilon_N))/(-\Delta\varepsilon_D - (-\Delta\varepsilon_{280}))$. From those data that can be assumed to provide reasonable estimates of K (in bold type), ΔG is calculated, according to $-RT\ln(K)$.

Temperature	Absorbance	Extrapolated Baselines		K	ΔG
(Kelvin)	$-\Delta\varepsilon_{280}$	$-\Delta\varepsilon_N$	$-\Delta\varepsilon_D$	[D]/[N]	(kcal)
281.74	0	2.374E-4	1.85159	-1.28214E-4	
290.76	0.008	0.00935	1.77898	-7.60934E-4	
294.62	0.009	0.01325	1.74791	-0.00244	
297.57	0.018	0.01623	1.72416	0.00104	
300.27	0.037	0.01895	1.70243	**0.01084**	2.69968
302.72	0.044	0.02143	1.6827	**0.01377**	2.5774
304.87	0.059	0.0236	1.6654	**0.02204**	2.31104
307.08	0.082	0.02583	1.64761	**0.03588**	2.03043
309.78	0.133	0.02856	1.62587	**0.06996**	1.63721
311.99	0.215	0.03079	1.60808	**0.13223**	1.25423
314.13	0.363	0.03295	1.59085	**0.2688**	0.82003
316.1	0.593	0.03494	1.575	**0.56829**	0.35495
318.3	0.911	0.03716	1.55729	**1.35209**	-0.19078
320.02	1.17	0.0389	1.54344	**3.02887**	-0.70468
322.23	1.355	0.04113	1.52565	**7.69926**	-1.30687
324.62	1.444	0.04355	1.50641	**22.43993**	-2.00656
326.58	1.467	0.04553	1.49063	**60.15294**	-2.7
330.27	1.459	0.04925	1.46093	731.76605	
333.64	1.437	0.05266	1.4338	-432.33716	
335.85	1.415	0.05489	1.41601	1349.9866	
339.53	1.385	0.05861	1.38638	958.72403	
343.15	1.36	0.06226	1.35724	-470.6214	

ΔG will be zero at the transition temperature (T_M). Inspection of the data in the table shows that T_M lies between 316.1 and 318.3 Kelvin. Linear interpolation is adequate to estimate T_M. **T_M = 317.5 Kelvin.**

The values of ΔG are plotted and fit to a third-order polynomial. The coefficients of the polynomial are used to estimate the thermodynamic quantities at T_M.

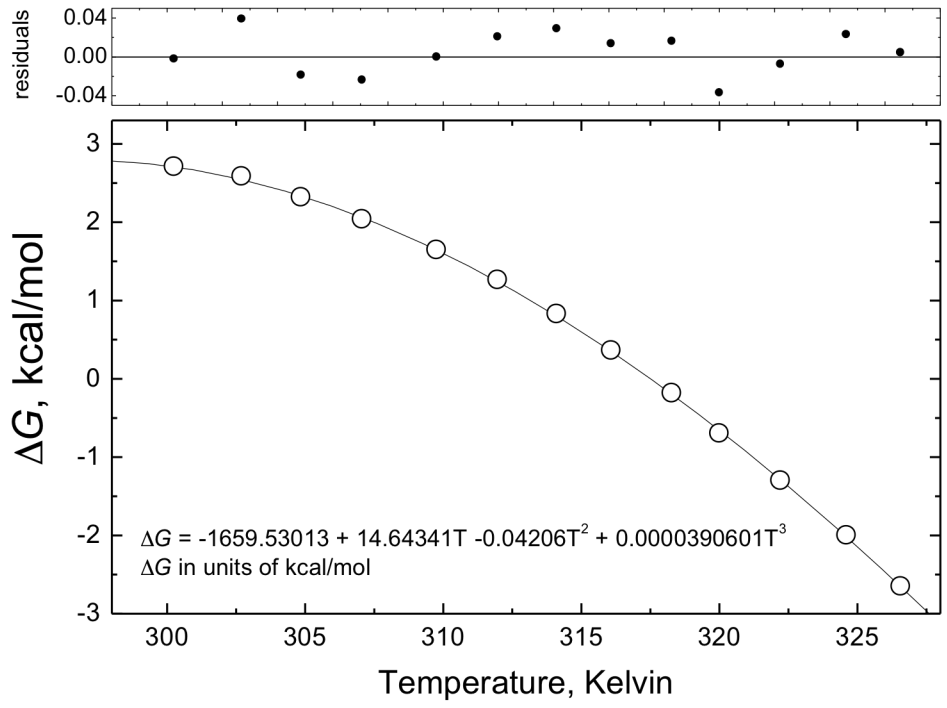
ΔH and ΔS are found by finding the derivatives of ΔG and $\Delta G/T$ using the polynomial definition for ΔG:

$$\Delta G = A + BT + CT^2 + DT^3$$

$$\frac{d(\Delta G)}{dT} = -\Delta S = B + 2CT + 3DT^2 \qquad \text{or} \qquad \Delta S = -B - 2CT - 3DT^2$$

$$\frac{d(\Delta G/T)}{dT} = -\frac{\Delta H}{T^2} \qquad \text{or} \qquad \Delta H = -T^2 \frac{d(\Delta G/T)}{dT}$$

$$\Delta H = -T^2 \frac{d(\Delta G/T)}{dT} = -T^2 \frac{d(A/T + B + CT + DT^2)}{dT} = A - CT^2 - 2DT^3$$



The Free Energy Change of denaturation *versus* temperature. The residuals (Δ*G*(exp) - Δ*G*(fit)) are plotted at the top.

Using these formulae the estimates for Δ*H* and Δ*S* at T_M (= 317.5 K), are

Δ*H* = -1659.53013 + 0.04206*(317.5)² - 2*3.90601*10⁻⁵*(317.5)³
Δ*H* = 80.1 kcal/mol (= 335 kJ/mol)

Δ*S* = -14.6434 + 2*0.04206*(317.5) - 3*3.90601*10⁻⁵*(317.5)²
Δ*S* = 252 eu (= 1.054 kJ/mol/deg)

ΔC_P of denaturation is estimated through the application of the formulae relating Δ*H* and/or Δ*S* to ΔC_P. *dH*/*dT* is the thermodynamic definition of C_P; and *d*S/*dT* = C_P/*T*. The application of either formula should lead to

$$\Delta C_P = -2CT - 6DT^2$$

and results in an estimate for ΔC_P of

ΔC_P = 2*0.04206*(317.5) - 6*3.90601*10^{-5}*(317.5)^2
ΔC_P = 3.1 kcal/mol/deg (= 13.0 kJ/mol/deg)

You should realize that the choice of points used to create the baselines can have a significant effect on the results. An alternative approach in fitting the data allows the baseline parameters to adjust simultaneously with the thermodynamic parameters, once the *form* for the baseline (*i.e.* constant, linear or curved) has been chosen.

To see this, start with the equation for the equilibrium constant, and solve it explicitly for the molar extinction coefficient, $\Delta\varepsilon$

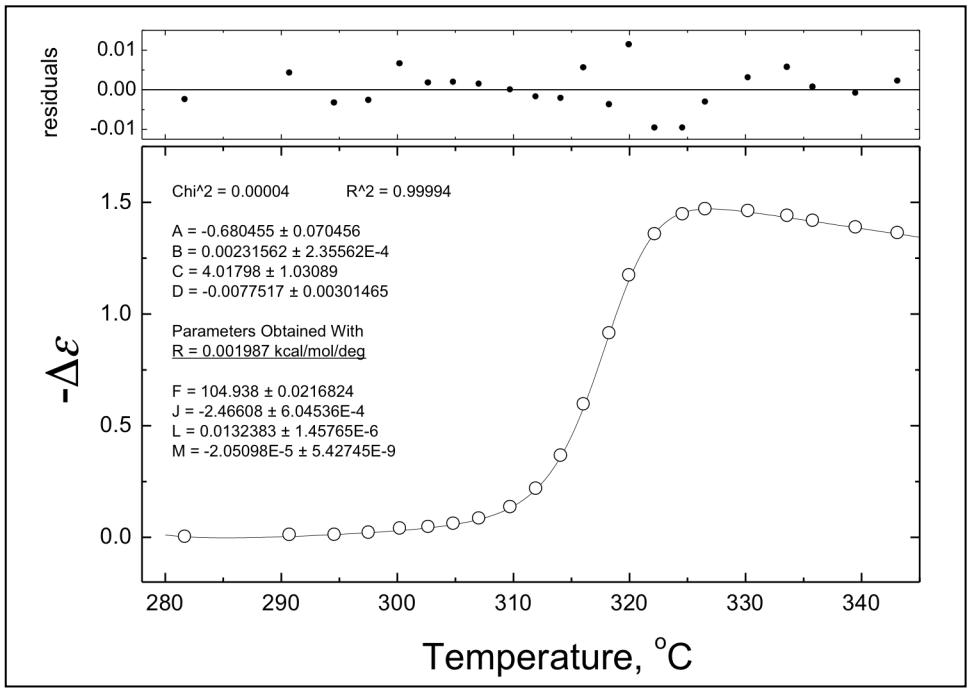
$$\Delta\varepsilon = \frac{\Delta\varepsilon_N + \Delta\varepsilon_D K}{1+K}$$

Then incorporate the formulae for each variable into this expression, *i.e.* substitute in

$$\Delta\varepsilon_N = A + BT \qquad \Delta\varepsilon_D = C + DT \qquad K = e^{-(F+JT+LT^2+MT^3)/RT}$$

to give

$$\Delta\varepsilon = \frac{C + DT + \left(A + BT\right)\cdot e^{-(F + JT + LT^2 + MT^3)/RT}}{1+e^{-(F + JT + LT^2 + MT^3)/RT}}$$



The coefficients ***A*** & ***B*** and ***C*** & ***D*** define the linear fits for the low, and high, temperature baselines, respectively, and ***F***, ***J***, ***L*** and ***M*** represent the coefficients in the power series approximation for ΔG. Reasonable initial values for a fitting session with this function are the values of the coefficients obtained by the (stepwise) procedure, outlined above. χ^2 is then minimized by adjusting all of the parameters ***A*** through ***J***. With this approach estimates for T_M, ΔH, ΔS and ΔC_P are:

Parameter	Value at T_M (317.5 K)	
	cgs	MKS
ΔH	83.3 kcal/mol	348.4 kJ/mol
ΔS	262.3 eu	1.098 kJ/mol/deg
ΔC_P	4.00 kcal/mol/deg	16.7 kJ/mol/deg

Part e. The temperature of maximum stability is given as the temperature at which the Gibbs energy function reached a maximum. This is computed as the temperature at which $d(\Delta G)/dT = 0$.

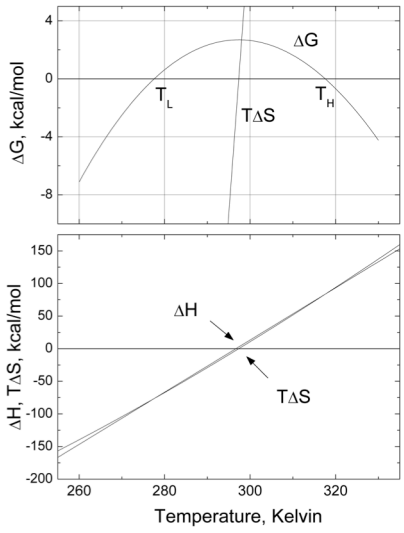
$$\frac{d(\Delta G)}{dT} = -\Delta S = 0 = -\left[\Delta S_R + \Delta C_P \ln\left(\frac{T}{T_R}\right)\right]$$

Solving for T, and using the parameters from the table above, gives:

$$T = T_R e^{-\Delta S_R / C_P} = 317.5\ x\ \exp\left[-0.2623/4\right] = 297K$$

Part f. A stability plot is, simply put, a graph of ΔG *versus* T

$$\Delta G = \Delta H_R + \Delta C_P (T - T_R) - T\left(\Delta S_0 + \Delta C_P \ln\left(\frac{T}{T_R}\right)\right)$$



Through an examination of function worksheet T_L, the cold denaturation temperature, is found to be 277 K.

Note that cold denaturation ($T_M = T_L$) is exothermic ($\Delta H_L < 0$) and also that $\Delta S_L < 0$! Is it reasonable to assume that the chain configurational entropy increases upon (cold) denaturation ($\Delta S_{L(config)} > 0$)? If yes, then the other factors contributing to the overall entropy change (ΔS_L) must be both larger ($|\Delta S_{L(other)}| > |\Delta S_{L(config)}|$) and negative ($\Delta S_{L(other)} < 0$).

Extra Credit Problem. *Carry out an analysis of DSC data for RNAse to determine,* T_M, ΔH_M, ΔS_M, ΔC_P, *the temperature of maximum stability, and the* T_M *of cold denaturation* (T'_G) *according to the manner of problem 1. In addition to the van't Hoff enthalpy change, calculate* ΔH_{cal}, *the calorimetric change in enthalpy.*

Conditions of the DSC Experiment
Scan Rate = 1 deg per minute
Cell Volume = 1.411 mL
[RNAse] = 63 μM

T_M = 60 ^oC, 333 K

Thermodynamic Parameters from the 'van't Hoff Analysis.'
Technically speaking, the analysis of the temperature-dependence of the Gibbs energy change (stability) is not a van't Hoff analysis. The van't Hoff equation, $d(\ln K)/d(T) = \Delta H/RT^2$, allows you to determine the enthalpy change of the reaction. This is informative. The more rapidly the equilibrium constant changes with temperature, the larger the enthalpy change – the analysis is based on the *shape* of the transition curve – *not* the area under it. The analysis of the temperature dependence of ΔG is also an analysis of K as a function of temperature, but provides estimates ΔS_M and ΔC_P in addition to ΔH_M. So both are based on changes in the reaction equilibrium with changes in temperature. (Equilibrium constants describe the relationship between the concentrations of reactants and products at constant temperature and pressure, but K changes when the temperature and/or pressure change.)

The steps of the analysis are:

(1) Subtract the buffer *vs.* buffer *reference* trace from the RNAse *vs.* buffer trace. In this case, the temperature values for the buffer *vs.* buffer data and protein *vs.* buffer traces are different. How to conduct a point-by-point subtraction? A fit of the reference trace to a polynomial provides a mechanism to create a buffer vs. buffer reference trace data set that has the same set of temperatures as the protein *vs.* buffer data.
(2) Integrate the reference-trace-subtracted data, to generate enthalpy vs. temperature data ($h(T)$).
(3) Construct low T and high T baselines: $h_L(T)$ and $h_D(T)$, respectively.
(4) Determine K ([D]/[N]) as a function of temperature, using $[h(T) - h_N(T)]/[h_D(T) - h(T)]$
(5) Convert K to ΔG via $-RT\ln K$.
(6) Determine T_M from inspection of the data ($\Delta G(T_M) = 0$).
(7) Select the appropriate sub-range in the $\Delta G(T)$ data set to fit. These values of $\Delta G(T)$ correspond to values of K that are determined most accurately, *e.g.* values between 0.05 and 20. There is some judgment exercised here, as long as the experimental observable is determined accurately, it is possible to use a wider K-range, *e.g.* 0.01 to 100. However, the differences, $h(T) - h_N(T)$ and $h_D(T) - h(T)$], are most accurate when $h(T)$ is not close in value to either $h_N(T)$ or $h_D(T)$ – that is, when K is in the neighborhood of 1 and ΔG is in the neighborhood of 0.

(8) Fit the $\Delta G(T)$ data to retrieve the thermodynamic values.
(9) Calculate T_{MAX} from $\Delta S_{MAX} = 0$, where $T_{MAX} = T_M \exp(-\Delta S_M/\Delta C_P)$.
(10) Use stability plots to determine the cold denaturation temperature.

Parameters derived from fit of $\Delta G(T)$ to $\Delta H_M + \Delta C_P(T - T_M) - T(\Delta S_M + \Delta C_P \ln(T/T_M))$
$\Delta H_M = 376.6 \pm 0.3$ kJ/mol
$\Delta S_M = 1.130 \pm 0.0008$ kJ/mol/degree
$\Delta C_P = 21.19 \pm 0.10$ kJ/mol/degree
$T_M = 333$ K (fixed)

When $\Delta S = 0$, $T = T_{MAX}$
$\Delta S_M + \Delta C_P \ln(T_{MAX}/T_M) = 0 \rightarrow T_{MAX} = T_M \exp(-\Delta S_M/\Delta C_P)$
$\boldsymbol{T_{MAX}}$ **=** $333\exp(-1.130/21.19) = 315.7$ K = **42.7 °C**

Parameters derived from fit of $\Delta G(T)$ to $A + B_1 T + B_2 T^2 + B_3 T^3$

$A = 4937 \pm 849$	4936.84418
$B_1 = -53.28 \pm 7.69$	-53.27863
$B_2 = 0.1898 \pm 0.0232$	0.18981
$B_3 = -2.232E\text{-}4 \pm 0.233E\text{-}4$	-2.23225e-4

It is well known in thermodynamics that $d(\Delta G)/dT = -\Delta S$
$\Delta G(T) = \Delta H_M + \Delta C_P(T - T_M) - T(\Delta S_M + \Delta C_P \ln(T/T_M)$
$\Delta G(T)/dT = -\Delta S(T)$
$\Delta G(T) = A + B_1 T + B_2 T^2 + B_3 T^3$
$\Delta S(T) = -B_1 - 2B_2 T - 3B_3 T^2$
$\Delta S_M = -B_1 - 2B_2(333) - 3B_3(333^2) = 53.27863 - 2(0.18981)(333) + 3(2.232E\text{-}4)(333^2)$
$\boldsymbol{\Delta S_M}$ **=** $53.27863 - 126.4135 + 74.2586$ = **1.124 kJ/mol/deg**

It is also well known in thermodynamics that $d(\Delta G/T)/dT = -\Delta H/T^2$
$\Delta G/T = A/T + B_1 + B_2 T + B_3 T^2$
$d(\Delta G/T)/dT = -A/T^2 + B_2 + 2B_3 T$
$\Delta H(T) = A - B_2 T^2 - 2B_3 T^3$
$\Delta H_M = 4936 - 0.1898(333^2) + 2(2.232E\text{-}4)(333^3)$
$\boldsymbol{\Delta H_M}$ **=** $4936.8 - 21047.8 + 16485.6$ **= 374.6 kJ/mol**

Finally, by definition, $d(\Delta H)/dT = \Delta C_P$
$\Delta H = A - B_2 T^2 - 2B_3 T^3$
$\Delta C_P = -2B_2 T - 6B_3 T^2$
$\Delta C_P = -2(0.1898)T - 6(2.232E\text{-}4)T^2$
$\boldsymbol{\Delta C_P} = -2(0.1898)333 - 6(2.232E\text{-}4)(333^2) = 126.4 - 148.52$ **= 22.12 kJ/mol/deg**

$T = T_{MAX}$ when $\Delta S = 0$
$\Delta S_M + \Delta C_P \ln(T_{MAX}/T_M) = 0 \rightarrow T_{MAX} = T_M \exp(-\Delta S_M/\Delta C_P)$
$\boldsymbol{T_{MAX}}$ **=** $333\exp(-1.124/22.12) = 316.5$ K = **43.5 °C**

Summary of Results

	*ΔG Function**	
Parameter	$\Delta H_M + \Delta C_P(T - T_M) - T(\Delta S_M + \Delta C_P \ln(T/T_M))$	$A + B_1T + B_2T^2 + B_3T^3$
T_M	60 °C	60 °C
ΔH_M	376.6 ± 0.3 kJ/mol	374.6 kJ/mol
ΔS_M	1.130 ± 0.0008 kJ/mol/deg	1.124 kJ/mol/deg
ΔC_P	21.19 ± 0.10 kJ/mol/deg	22.12 kJ/mol/deg
T_{MAX}	42.7 °C	43.5 °C

*The uncertainties in the parameters were estimated in Origin using error matrix calculations. Uncertainties in the parameters according to the polynomial fit were not calculated, but could have been estimated by propagation of errors.

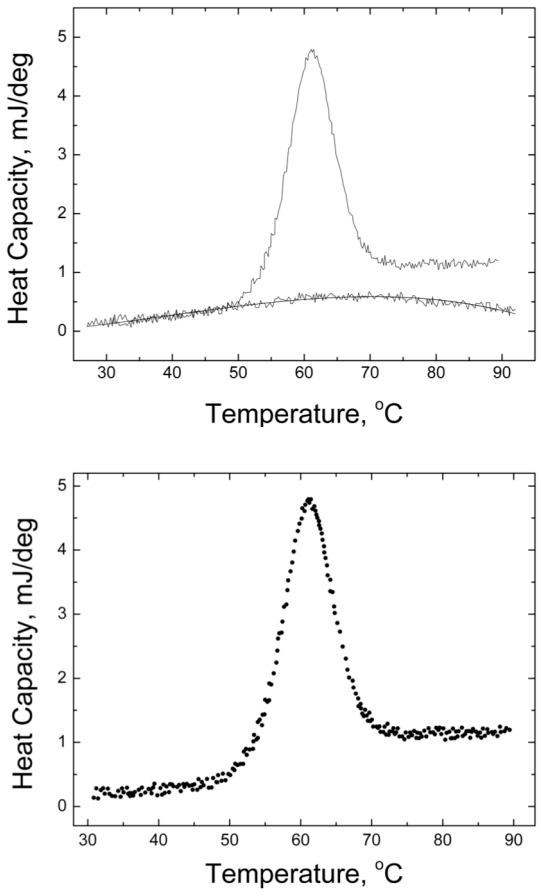


Figure EC1. Top: Heat capacities of protein vs. buffer and buffer vs. buffer. Bottom: protein vs. buffer with reference trace subtracted.

The experimental heat capacity trace of the RNAse solution is marked by a transition. Below the transition, the heat capacity has a contribution from folded protein. Above the transition, the heat capacity has a contribution from denatured protein. The peak, which defines the transition region, reflects the excess heat capacity of the system. The buffer *vs.* buffer trace (no peak) is a *reference* trace (*not a baseline*). The curvature reflects the behavior of the instrument (long-term drift), close to the performance limit.

The subtraction of the reference trace, fortuitously removes the curvature in the baselines on either side of the transition. The trace has a positive ΔC_P (= C_P(denatured) – C_P(native) > 0). More subtle is the fact that the slopes in the baseline above and below the transition are different. This implies that ΔC_P will vary with temperature. (Why? Only lines that are parallel never intersect. If the slopes are different, the lines are not parallel.)

Integration of the reference-trace subtracted data gives the enthalpy as a function of temperature (Figure EC2, top). Using the low and high temperature regions to create and extrapolate baseline behavior in the transition region, permits the calculation of *K* in the manner that was applied to the difference UV-Vis experiment in problem 1.

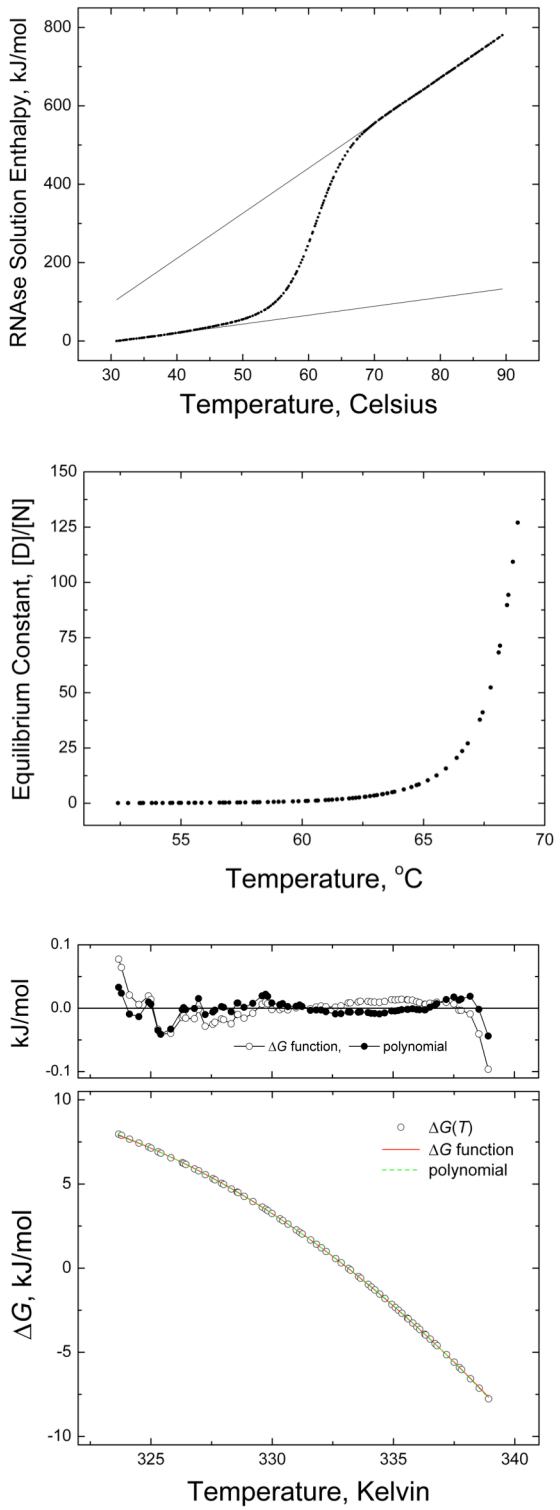


Figure EC2. **Top:** enthalpy vs. *T*; **2nd from Top:** *K vs. T,* determined from the enthalpy; **Bottom:** Fit of ΔG *vs. T* to two models; **3rd from Top**: residuals from the two fits.

The enthalpy is drawn at the left with the low and high temperature baselines. The equilibrium constant is calculated as a function of temperature according to

$$K = [h(T) - h_N(T)]/[h_D(T) - h(T)]$$

In the plot at the left, the values of K far from the midpoint (K = 1) have already been removed.

The values of K were further restricted to include values between 0.05 and 20, the values of K that are most accurately estimated.

This range corresponds to changes in ΔG^o between 8 and -8 kJ/mole:

$$\Delta G^o = -RT\ln(0.05)$$
$$\Delta G^o = -(0.008314 \text{ J/mol/deg})(333)\ln(0.05)$$
$$\Delta G^o = -8.29 \text{ kJ/mol/deg})$$

$$\Delta G^o = -RT\ln(20)$$
$$\Delta G^o = -(0.008314 \text{ J/mol/deg})(333)\ln(20)$$
$$\Delta G^o = -(8.314 \text{ J/mol/deg})(333)\ln(0.05)$$

Two different models, the ΔG function that assumes ΔC_P is constant, and a power series in T.

$$\Delta G(T) = \Delta H_M + \Delta C_P(T - T_M) - T(\Delta S_M + \Delta C_P \ln(T/T_M)$$

$$\Delta G(T) = A + B_1 T + B_2 T^2 + B_3 T^3$$

The plot of the residuals indicates that both functions fit the data reasonably well. The fit of the data to the ΔG function provides estimates of the thermodynamics directly. A second, generic method, to fit the data to a polynomial, and use the coefficient to arrive at estimates of the thermodynamics parameters, yields similar results. (See table above.)

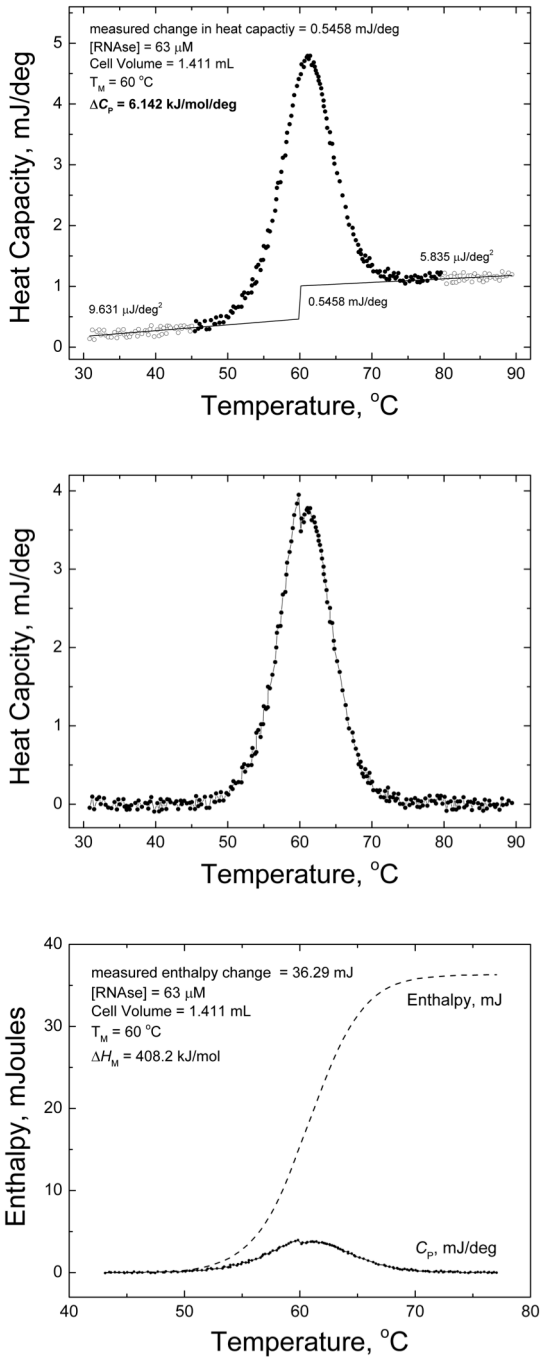


Figure EC3. Top: The determination of ΔC_P depicts the *reference-trace*-subtracted DSC curve with linear baselines extended into the transition region. Middle: DSC data with baselines *and* the reference trace subtracted. Bottom: The area under the DSC traces gives the enthalpy of unfolding for the RNAse sample (mJ).

Thermodynamic Parameters Estimated from the Calorimetry Experiment. Estimates of ΔC_P, ΔH_M & ΔS_M can be made directly with a calorimetric measurement. Calorimetry is the only method that gives two independent estimates of ΔH (van't Hoff and calorimetric).

ΔC_P can be estimated with the procedure illustrated in the textbook (Ch. 5): the low & high *T* baselines modeled as lines, which are extended into the transition region. At the transition temperature (60 °C), the difference in the heat capacity of native and denatured protein baselines gives an estimate of ΔC_P.

The experimental estimate, 0.5458 mJ/deg, is converted to ΔC_P, the molar quantity, using the moles of protein in the calorimeter cell.

moles RNAse = molar conc. x cell volume
= 63e-6 M *x* 1.411e-3 L
= 88.893e-9 moles

ΔC_P = (0.546e-6 kJ/deg)/(88.893e-9 moles)
ΔC_P = 6.142 kJ/mol/deg

The calorimetric estimate of ΔC_P is smaller than the estimate from the van't Hoff analysis.

ΔH_M(cal) is estimated by subtracting the baseline (in Figure EC3, top) from the DSC trace to give the baseline-subtracted trace (middle). Integration of the baseline-subtracted peak is the experimental value for the unfolding enthalpy (mJ). Like ΔC_P, ΔH_M(cal) (kJ/mol) is determined by dividing through the number of moles in the sample.

Δh = 36.29 mJ (integrated area)
ΔH_M(cal) = (36.29 mJ)/(88.893e-9 moles)
ΔH_M(cal) = 408.2 kJ/mol

Finally, **ΔS_M(cal)** is calculated as

ΔS_M(cal) = ΔH_M(cal)/T_M
ΔS_M(cal) = (408.2 kJ/mol)/(333K)
ΔS_M(cal) = 1.226 kJ/mol/deg

The DSC experiment is unique in providing two independent estimates of ΔH, ΔH_{vH} through the analysis of the change in K as a function of temperature, and ΔH_{cal}, by direct integration of the heat capacity trace. The value of ΔH_{vH} depends on the nature of the reaction equilibrium that is assumed for the unfolding process. The value of ΔH_{cal} is *model-independent*. If the reaction equilibrium chosen to model the unfolding process does not match the actual process, then ΔH_{vH} will not equal ΔH_{cal}. The ratio between van't Hoff and calorimetric ratios becomes a test of whether the model of the reaction equilibrium correctly describes the unfolding events. In this case we assume a simple two-state process involving the equilibrium between monomers of folded and denatured RNAse. The ratio

$\Delta H_M(vH)/ \Delta H_M(cal) = 375.6/408.2 = 0.92 \sim 1$

is close to 1, which provides evidence that RNAse is unfolding in the DSC experiment, without intermediates, and/or without additional cooperativity through oligomerization. Starting at the bottom of page 9 on the Protein Stability handout, two state unfolding processes coupled to oligomerization is analyzed with a mathematical model. It is apparent that the shape of the simulated DSC trace changes as a function of the degree of oligomerization, despite the fact that the area under the curve does not. In this situation $\Delta H_{vH} \neq \Delta H_{cal}$ when the equilibrium process used in the van't Hoff is different than the process that takes place in the calorimeter cell.