A Thermodynamic Molecular Switch in Biological Systems: Ribonuclease S′ Fragment Complementation Reactions

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ABSTRACT It is well known that essentially all biological systems function over a very narrow temperature range. Most typical macromolecular interactions show $\Delta H°(T)$ positive (unfavorable) and a positive $\Delta S°(T)$ (favorable) at low temperature, because of a positive ($\Delta Cp°/T$). Because $\Delta G°(T)$ for biological systems shows a complicated behavior, wherein $\Delta G°(T)$ changes from positive to negative, then reaches a negative value of maximum magnitude (favorable), and finally becomes positive as temperature increases, it is clear that a deeper-lying thermodynamic explanation is required. This communication demonstrates that the critical factor is a temperature-dependent $\Delta Cp°(T)$ (heat capacity change) of reaction that is positive at low temperature but switches to a negative value at a temperature well below the ambient range. Thus the thermodynamic molecular switch determines the behavior patterns of the Gibbs free energy change and hence a change in the equilibrium constant, K_{eq}, and/or spontaneity. The subsequent, mathematically predictable changes in $\Delta H°(T)$, $\Delta S°(T)$, $\Delta W°(T)$, and $\Delta G°(T)$ give rise to the classically observed behavior patterns in biological reactivity, as may be seen in ribonuclease S′ fragment complementation reactions.

INTRODUCTION

Shortly after T. H. Benzinger published a paper in 1971 titled "Thermodynamics, Chemical Reactions and Molecular Biology," the question arose whether thermochemical descriptions of biochemical systems in the literature were inadequate or wrong or both.

After years of detailed study on interacting protein systems and their thermodynamic parameters, it is possible to formulate a viewpoint of thermodynamic reactions that is both adequate and consistent when applied to biological systems, and which considers the innate thermodynamic quantities in structural biology.

As made apparent in Benzinger's pioneering studies, in any chemical reaction involving small molecules, differences in heat capacities between products and reactants could be considered negligible, and consequently, the heat of reaction consists primarily of the contribution from the chemical bonding term and, therefore, is a close approximation of the chemical bond energy. Thus many scientists are comfortable thinking about reaction enthalpy in terms of "bonds formed minus bonds broken," that is, based upon the difference in bond energies between products and reactants.

In the case of biological interactions, Benzinger observed that the difference between the heat capacities of products and reactants may be substantial enough to totally obscure any difference between the chemical bonding term and the heat capacity integral. Thus the heat of reaction as traditionally defined cannot be used to accurately represent the chemical forces associated with these systems.

Received for publication 15 July 1999 and in final form 27 August 1999.
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0006-3495/00/01/416/14 $2.00

It has been well established in pure and applied chemistry of simple molecules that reaction energies at room temperature can be understood in terms of two contributions, one related to energy differences at 0 K (the innate inherent bond energy) and the other associated with integrals of heat capacity data over a range of temperatures (the thermal agitation energy). The necessity of a comparable separation of energy terms for biological reactions, however, has not been obvious to most workers in structural and molecular biology. The innate thermodynamic quantities (Gibbs, 1878; Planck, 1927; Moelwyn-Hughes, 1957; Lewis and Randall, 1961), particularly the innate temperature-invariant enthalpy, represent differences in chemical bonding energy of products minus reactants and thus control the heat of reaction.

In biological systems, which function over a very narrow temperature range, the standard Gibbs free energy change, $\Delta G°$, shows a complicated behavior, changing from positive to negative, then reaching a negative (and thus favorable) minimum, before finally becoming positive as temperature increases.

It is reasonable to search for an underlying thermodynamic explanation for the greater complexity known to occur in typical biological systems. This communication proposes that the critical factor driving this process is a temperature-dependent heat capacity change of reaction, which is positive at low temperature but switches to a negative value at a temperature well below the ambient range. This thermodynamic molecular switch determines the behavior patterns of the Gibbs free energy change, and hence a change in the equilibrium constant, K_{eq}, and/or spontaneity. The subsequent, mathematically predictable changes in $\Delta H°$, $\Delta S°$, $\Delta W°$, and $\Delta G°$ that arise as a result of this thermodynamic molecular switch are demonstrated in RNase S′ fragment complementation reactions.

THE INNATE THERMODYNAMIC QUANTITIES

The Gibbs free energy, or more correctly, the change in partial molar free energy for the components in a reaction mixture, undoubtedly does provide the correct thermodynamic criterion of equilibrium. Furthermore, it is often desirable to relate the drive toward equilibrium to a structural aspect of a system, that is, to understand the underlying factors that contribute to the change in ΔG° as a function of temperature for a specific reaction.

Certain components of the total change in ΔG° may be described as "innate," that is, the quantity ΔG° has an extrapolated value even at absolute zero temperature. There are also components that change with temperature. We note, for example, that at $T = 0$ K, $T\Delta S^\circ = 0$. Accordingly, because $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ (Gibbs, 1978; Planck, 1927; Moelwyn-Hughes, 1957; Lewis and Randall, 1961), it is found that at absolute zero K, $\Delta H^\circ_0 = \Delta G^\circ_0$ ($\Delta H^\circ(T_0) = \Delta G^\circ(T_0)$ in some texts). The residual values of these quantities (note that entropy is excluded) are the same at absolute zero of the temperature scale and may be said to describe the innate thermodynamic stability of the system.

For a chemical reaction, the difference in these quantities between reactants and products, that is, $\Delta H^\circ_0 = \Delta G^\circ_0$, represents the differences in the innate thermodynamic stability between reactants and products, or the change in stability between reactants and products as the reaction occurs.

THE GIAUQUE FUNCTION AND PLANCK-BENZINGER THERMAL WORK FUNCTION

One form of the free energy function, the Giauque function (Giauque, 1930a,b; Giauque and Blue, 1930; Giauque and Kemp, 1938; Giauque and Meads, 1941), $(G_0^\circ - H_0^\circ)/T = -\Psi^\circ/T$, where $\Psi^\circ = G^\circ_T - H^\circ_0$, has been extensively used in chemistry and physics. An equivalent formulation has recently found application in the biochemical literature as the Planck-Benzinger thermal work function (Chun, 1988, 1994, 1996b, 1997a, 1998), where the application to a given situation is quite different. Here the Planck-Benzinger thermal work function, $\Delta W^\circ(T) = \Delta H^\circ(T_0) - \Delta G^\circ(T)$, represents the strictly thermal components of any intra- or intermolecular bonding term, that is, energy other than the inherent difference in the 0 K portion of the interaction energy. The latter is the only energy term in constant pressure processes at absolute zero Kelvin. Thus $\Delta W^\circ(T)$ expresses completely the thermal energy difference of the process involved. Application of the thermal work function permits the separation of 0 K energy differences and energy differences associated with heat capacity integrals for a fuller understanding of reaction energies.

Measuring enthalpy values

Enthalpies of reaction are frequently measured at or near room temperature (298 K) for a variety of theoretical and practical reasons, for instance, the relationship between $\Delta H^\circ_{\text{reaction}}$ and the temperature coefficient of the equilibrium constant, K_{eq}, $\mathrm{d}\ln K_{eq}/\mathrm{d}(1/T) = -\Delta H^\circ(T)/R$. Kirchhoff's law (Moelwyn-Hughes, 1957; Lewis and Randall, 1961) states that $\Delta H^\circ_{298} = \Delta H^\circ(T_0) + \int_0^{298} \Delta Cp\, \mathrm{d}T$, where this last term represents the thermal agitation energy (heat capacity integrals), while the constant term $\Delta H^\circ(T_0)$ represents the enthalpy of reaction at 0 K. For small molecules, reaction enthalpies are often obtained around room temperature, and the heat of reaction is estimated in terms of the innate temperature-invariant enthalpy (inherent chemical bond energy), $\Delta H^\circ(T_0)$. (The innate temperature-invariant enthalpy, $\Delta H(T_0)$ (or in some texts, ΔH_0), is an equivalent terminology for this quantity in the inherent 0 K enthalpy (innate thermodynamic quantity). It represents the chemical bond energy difference corrected to 0 K.)

T. L. Cottrell (1958) pointed out 40 years ago that ΔH°_{298} and $\Delta H^\circ(T_0)$ differ only by ~1% in small molecules, but in 1971 T. H. Benzinger made the crucial observation that this difference is large in biological macromolecules because of the large magnitude of the heat capacity integrals (thermal agitation energy). In other words, for small molecules, $\Delta H^\circ_{298} - \Delta H^\circ(T_0)$ is a correction of only a few percent, whereas for biological macromolecules, the heat capacity integrals can be large, from 10% to 90% of the total heat of reaction. At present the scientific literature provides no "silver bullet," that is, no highly accurate method for evaluating $\Delta H^\circ_{298} - \Delta H^\circ(T_0)$ in large biological macromolecules; however, Chun's work (1988, 1994, 1995, 1996b, 1997a, 1998) has extensively addressed the problem.

METHODS AND COMPUTATIONAL PROCEDURES

To analyze the thermodynamic processes operating in a particular biological system, it is necessary to extrapolate the thermodynamic parameters over a much broader temperature range. The enthalpy, entropy, and heat capacity terms are evaluated as partial derivatives of the Gibbs free energy function defined by Helmholtz-Kelvin's expression (Moelywn-Hughes, 1957; Lewis and Randall, 1961):

$$\partial\Delta G(T)/\partial T = -\Delta S(T), \quad \{\partial\Delta G(T)/T\}/\partial T = -\Delta H(T)/T^2$$

$$\partial\Delta H(T)/\partial T = \Delta Cp(T), \quad \partial\Delta S(T)/\partial T = \Delta Cp(T)/T$$

In continuing studies on dozens of interacting protein systems, it has been shown in our laboratory that the third-order polynomial function provides a good fit in the temperature range accessible in biochemical systems (Chun, 1988, 1994, 1997a, 1998). In fact, it is shown to be correct in the low-temperature limit. The rationale for selecting the linear and nonlinear third-order (T^3 model) polynomial functions for $\Delta G^\circ(T) = \alpha + \beta T^2 + \gamma T^3$ (macromolecular interactions) and $\Delta H(T) = \alpha + \beta T^3 e^{\gamma T}$ (macromolecular unfolding) is found in the fundamentals of relevant quantum

theory (Planck, 1927; Moelywn-Hughes, 1957). At low temperatures, the specific heat of a simple solid becomes $C_V = (12\pi^4/5)(N_0K)(kT/h\upsilon_m)^3$. With a proper substitution of $\theta = h\upsilon_m/k$ and $R = N_0k$, $C_v = (12\pi^4R/5)(T/\theta)^3$ (Moelywn-Hughes, 1957; Lewis and Randall, 1961). Clearly, the energy and specific heat are universal functions of $(kT/h\upsilon_m)$ or (T/θ). Here k is the Boltzmann constant, C_V is the specific heat at constant volume, and υ_m is the maximum frequency of vibration of an atom in a solid state, as in Planck's theory (Planck, 1927).

Determination of the Gibbs free energy change as a function of temperature

1. The binding constants, K_B (M^{-1}), as a function of temperature, were evaluated from the calorimetric titration of the S-protein of RNase S with various substitutions at Met^{13}-S-peptide, as reported by Naghibi et al. (1995). We converted these binding constants into the Gibbs free energy change as a function of temperature.
2. The Gibbs free energy data for the fragment complementation reactions of S-peptide with S-protein and of $Met(O_2)^{13}$-S-peptide with S-protein were also extracted from figures 4 and 5, respectively, of Hearn et al. (1971).
3. The data for the Gibbs free energy change of thermal unfolding of four mutants were extracted from figures 3 and 4 of Connelly et al. (1991), using the expression ΔG°_d (mutant) = $\{\Delta G^\circ_d(WT) - \Delta G^\circ_d\}$, where $\Delta G^\circ_d(WT)$ as a function of temperature was evaluated from figure 2 of Connelly et al. (1991).

Computational procedure for macromolecular interactions

(The practical use of Benzinger's method (1971) requires "the experimental determination of the all-important zero-point enthalpy and free entropy terms" pointed out by Rhodes (1991). As noted by Rhodes, Benzinger was pessimistic about the prospects for practical use of his method, due to the difficulty of obtaining the required (temperature-invariant) quantities. Our solutions to this difficulty are addressed in the present series of papers (Chun, 1988, 1994, 1995, 1996a,b, 1998).) The approach that we follow requires exact determination of K_{eq} for the relevant biological processes as a function of absolute temperature. Of critical importance, however, is the calculation of $\Delta Cp^\circ_{reaction}$ as a function of temperature as shown in Eq. 3. In this treatment, the Gibbs free energy data, as shown in Figs. 1 and 2, *A–D*, were fitted to a three-term linear polynomial function in the 273–320 K temperature range, the range in which experiments have been conducted:

$$\Delta G^\circ(T) = \alpha + \beta T^2 + \gamma T^3 \quad (1)$$

Once evaluated as shown in Figs. 1 and 2, *A–D*, the coefficients α, β, and γ were fitted to other thermodynamic

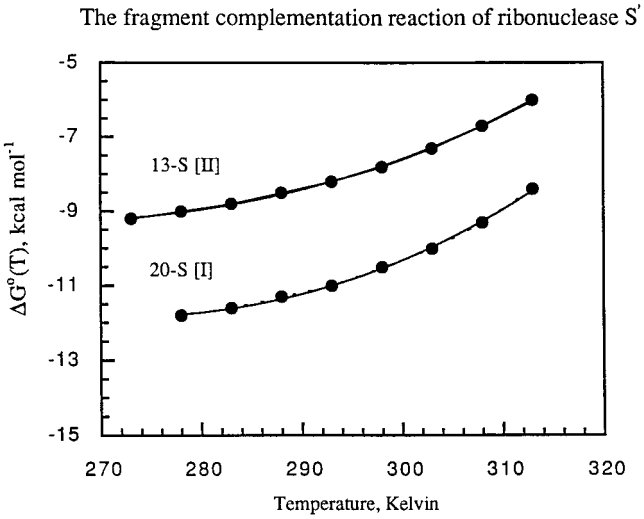

FIGURE 1 Thermodynamic plots of the standard Gibbs free energy change of the fragment complementation reaction of a ribonuclease S′ system as a function of temperature in the temperature range of 273–313 K, at pH 7.0 in 0.3 M NaCl based on data reported by Hearn et al. (1971). The experimental data for equilibrium and calorimetric measurements were evaluated with the general linear model procedure of statistical analysis of the IMSL subroutine. The solid line represents fitted data. $F = 0.001$; thus the goodness of fit of the experimental data was 99.9% or better in each case. The expansion coefficients α, β, and γ for 20S-RNase S′ and 13S-RNase S′ were (Chun, 1997a) as follows:
20-S[I], formation of 20S-RNase S′:
$\alpha = 34.05 \pm 1.30$ kcal mol^{-1}; $\beta = -1.856 \times 10^{-3}$ kcal mol^{-1} K^{-2}; $\gamma = 4.5447 \times 10^{-6}$ kcal mol^{-1} K^{-3}; $R^2 = 0.9995$; SD = 0.01342; PR > $F = 0.0001$.
13-S[II], formation of 13S-RNase S′:
$\alpha = 19.49 \pm 1.08$ kcal mol^{-1}; $\beta = -1.2034 \times 10^{-3}$ kcal mol^{-1} K^{-2}; $\gamma = 3.0989 \times 10^{-6}$ kcal mol^{-1} K^{-3}; $R^2 = 0.990$; SD = 0.02436; PR > $F = 0.0002$.

parameters. $\Delta H^\circ(T)$, $\Delta Cp^\circ(T)$, $T\Delta S^\circ(T)$, and $\Delta W^\circ(T)$ are defined as follows:

$$\Delta H^\circ(T) = \alpha - \beta T^2 - 2\gamma T^3 \quad (2)$$

$$\Delta Cp^\circ(T) = -2\beta T - 6\gamma T^2 \quad (3)$$

$$T\Delta S^\circ(T) = -2\beta T^2 - 3\gamma T^3 \quad (4)$$

$$\Delta W^\circ(T) = -\beta T^2 - \gamma T^3 \quad (5)$$

To extrapolate down to 0 K, it is necessary to consider normal solution states of macromolecules. Here the 0 K limit would presumably refer to the glassy condition (Chun, 1996a), that is, a condition with all thermal agitation frozen out, but retaining the general physical chemical properties of solution—since a pure crystalline form of macromolecules is rarely encountered in practice. (Why is Helmholtz-Kelvin's expression considered to be a continuous function? Our evaluation of the innate temperature-invariant enthalpy for hydrogen-bonded water in equilibrium with non-hydrogen-bonded water molecules is based on Helmholtz free energy data reported by Nemethy and Scheraga [1962]. The

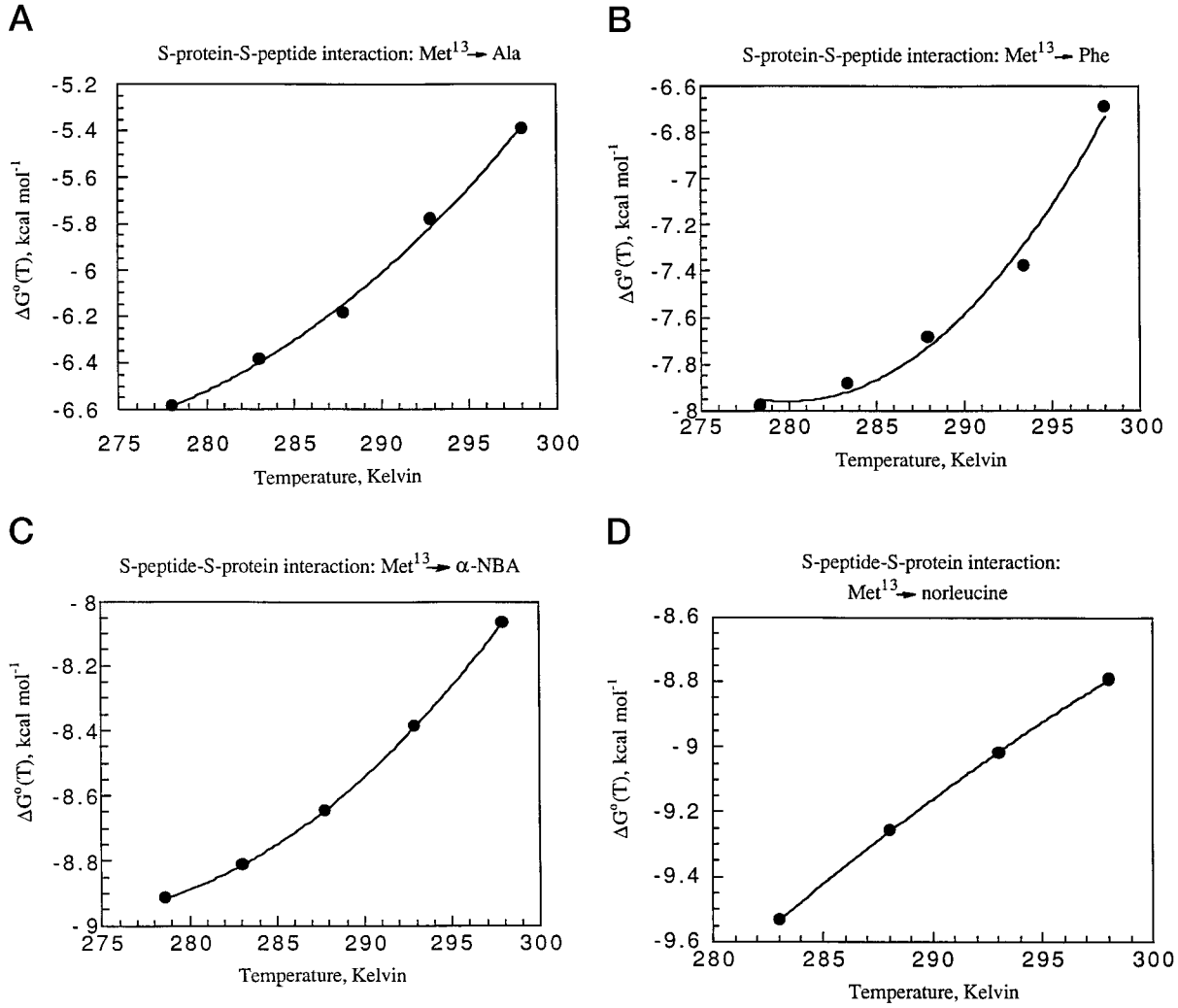

FIGURE 2 Thermodynamic plot of the nonstandard Gibbs free energy change of the fragment complementation reaction of the S-protein of RNase S′ with various substitutions at Met^{13} S-peptide as a function of temperature, based on data reported by Naghibi et al. (1995) and by Varadarajan et al. (1992) in the temperature range of 288–303 K in 50 mM sodium acetate with 100 mM NaCl, pH 6.0. The experimental data for differential scanning calorimetry were evaluated with the general linear model procedure of statistical analysis of the IMSL subroutine. The solid line represents fitted data. $F = 0.001$; thus the goodness of fit of the experimental data was 99.9% or better in each case. The expansion coefficients α, β, and γ for various substitutions at Met^{13} as a function of temperature (Chun, 1997a) were as follows.

(*A*) M13A RNase S′: $\alpha = 25.05 \pm 0.36$ kcal mol^{-1}; $\beta = -1.358 \times 10^{-3}$ kcal mol^{-1} K^{-2}; $\gamma = 3.384 \times 10^{-6}$ kcal mol^{-1} K^{-3}; $R^2 = 0.9998$; SD $= 4.122 \times 10^{-4}$; PR $> F = 0.001$.

(*B*) M13F RNase S′: $\alpha = 85.68 \pm 1.43$ kcal mol^{-1}; $\beta = -3.587 \times 10^{-3}$ kcal mol^{-1} K^{-2}; $\gamma = 3.384 \times 10^{-6}$ kcal mol^{-1} K^{-3}; $R^2 = 0.0934$; SD $= 0.0143$; PR $> F = 0.0$.

(*C*) M13α-NBA RNase S′: $\alpha = 22.32 \pm 0.78$ kcal mol^{-1}; $\beta = -1.269 \times 10^{-3}$ kcal mol^{-1} K^{-2}; $\gamma = 3.111 \times 10^{-6}$ kcal mol^{-1} K^{-3}; $R^2 = 0.9994$; SD $= 5.494 \times 10^{-4}$; PR $> F = 0.001$.

(*D*) M13Norleucine RNase S′: $\alpha = -33.52 \pm 0.78$ kcal mol^{-1}; $\beta = 6.997 \times 10^{-4}$ kcal mol^{-1} K^{-2}; $\gamma = -1.403 \times 10^{-6}$ kcal mol^{-1} K^{-3}; $R^2 = 0.9999$; SD $= 1.6319 \times 10^{-5}$; PR $> F = 0.001$.

entropy of the system appears to remain independent of temperature, suggesting that there is no significant temperature-dependent difference in the degree of orientation between unbound and hydrogen-bound water molecules in equilibrium in the system. A similar conclusion could be reached based on the dielectric relaxation of water as a function of temperature as reported by Collie et al. [1948]. This implies that there is a nonzero entropy difference for the transformation between water monomer and *n*-mer at zero Kelvin. As with most small molecules, the thermal agitation energy is minimal. $\Delta W(T) = T\Delta S(T)$, $\Delta H(T) = \Delta H(T_0)$ over the entire temperature range from 0 K to the temperature of interest. $\Delta S(T)$ obtained from Helmholtz free energy data and $\Delta S(T)$ from dielectric relaxation data remain constant and have values of 17.44 cal mol^{-1} deg^{-1} and 4.98 cal mol^{-1} deg^{-1}, respectively. These values are

independent of temperature, and can be extrapolated to 0 K [unpublished results] [Chun, 1996a]. There may be a small finite $\Delta S°(T_0)$ or $\Delta S°_0$ of formation. For these dilute systems of macromolecules, the $\Delta Cp°/T$ is largely controlled by the solvent water. As already noted, the assumed reference condition is glassy ice at 0K, which probably does have a small $\Delta S°_0$. In the case of $\Delta G°(T)$ of formation, phase transition is indeed important. When dealing with $\Delta G°(T)$ of reaction, no phase transition is taking place. In this case, all thermodynamic functions are continuous.)

Values of these thermodynamic parameters were regenerated from the fitted coefficients of α, β, and γ, using the International Mathematical Subroutine Library (IMSL) software for linear and nonlinear polynomial regression analysis. Each equation was interactively executed in steps of one K, and the values were plotted and overlaid for each set of experimental conditions. This IMSL program was incorporated into software for the computer-aided analysis of biochemical processes, and each data point was evaluated with extrapolation of F-statistics with an IBM personal computer (Chun, 1991; Barr et al., 1985), as shown in Figs. 3, *A* and *B*, 4, *A–C*, and 5, *A–C*.

A built-in restriction in the extrapolation procedure is that the values for $\Delta H°(T)$ and $\Delta G°(T)$ determined from the polynomial functions intersect at zero Kelvin with zero slope on a thermodynamic plot, thus obeying Planck's definition of the Nernst heat theorem (Moelwyn-Hughes, 1957; Lewis and Randall, 1961). By our definition, the value of $\Delta H°(T_0)$ will be positive. Other polynomial functions failed to meet all three restrictions of $\Delta H°(T)$ and $\Delta G°(T)$ intersecting at zero Kelvin with zero slope, and $\Delta H°(T_0)$ being positive and thus were discarded.

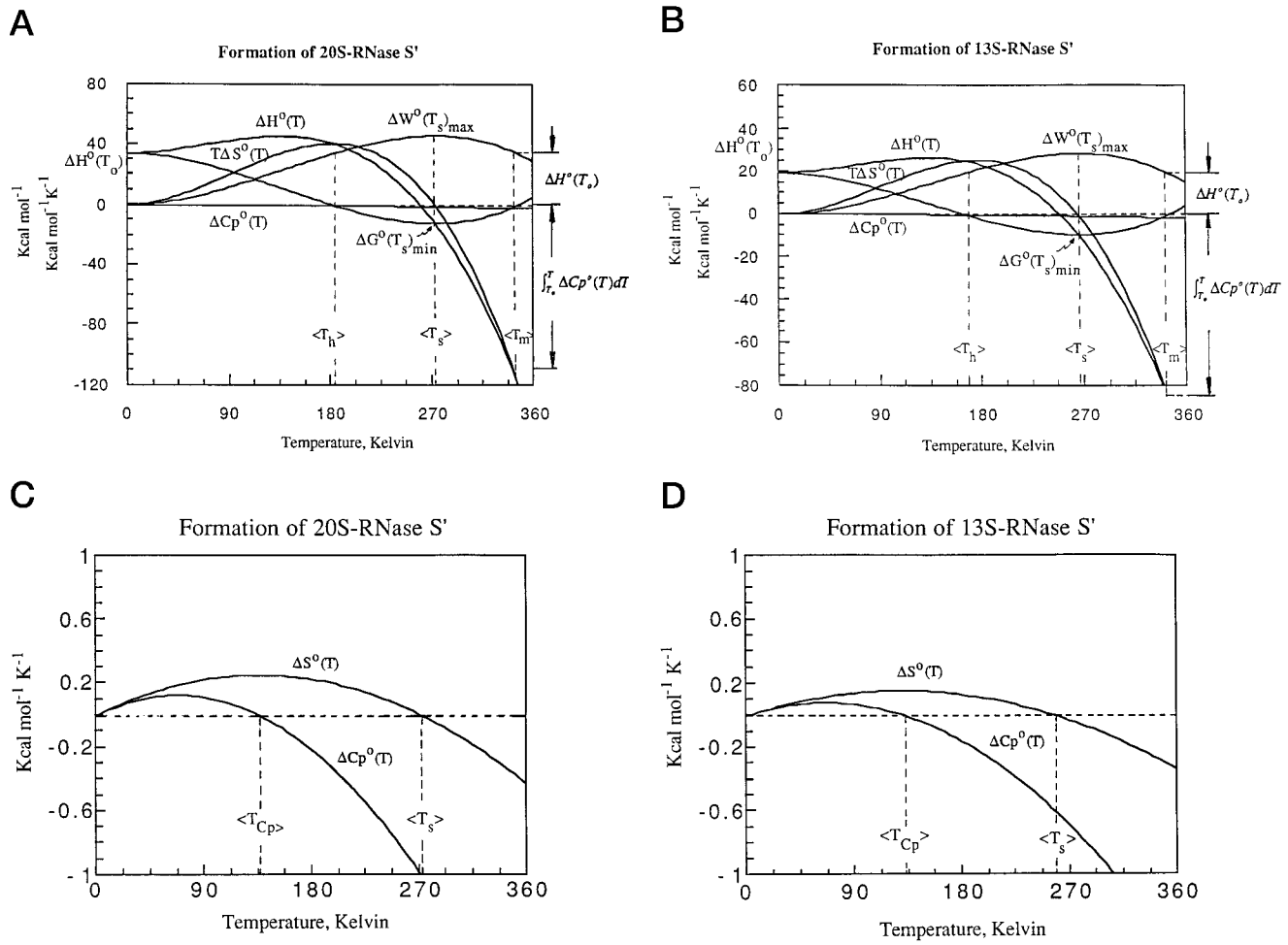

FIGURE 3 Thermodynamic plots of the fragment complementation reaction of 20-S [I] RNase S′ (*A*) and of 13-S [II] RNase S′ (*B*) as a function of temperature at pH 7.0 in 0.3 M NaCl. Each data point between 0 and 360 K was evaluated with extrapolation of F-statistics. Data points below 273 K were extrapolated from the experimental data. (Reprinted from Chun, P.W., 1997a, *J. Phys. Chem.* 101B: 7835. ©1997, American Chemical Society.) (*C* and *D*) A close-up view of a portion of 20-S [I] RNase S′ (*C*) and of 13-S [II] RNase S′ (*D*) over the temperature range of 0 K to 360 K, with the magnitude of the *y*-axis reduced to 1.0 to −1.0 kcal mol^{-1} K^{-1}. Thermodynamic molecular switch occurs when $\Delta Cp°(T)$ at $\langle T_{Cp}\rangle = 135$ K changes sign from positive to negative at $\langle T_s\rangle = 270$ K. $\Delta Cp°(T)$ and $\Delta S°(T)$ reach a maximum at 65 K and 135 K, respectively.

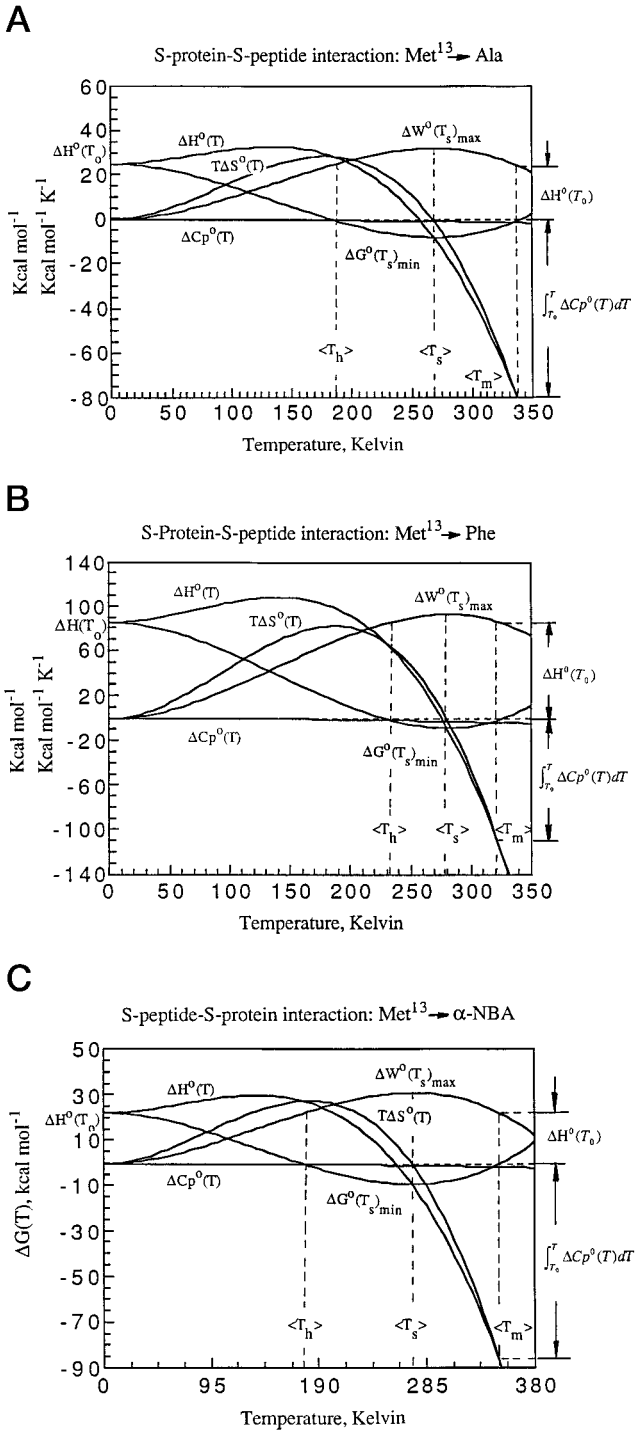

FIGURE 4 Thermodynamic plots of the fragment complementation reaction of S-protein of RNase S′ with various substitutions at Met^{13} of S-peptide as a function of temperature in 50 mM sodium acetate with 100 mM NaCl, pH 6.0. Each data point between 0 and 380 K was evaluated with extrapolation of F-statistics. (*A*) M13A RNase S′. (*B*) M13F RNase S′. (*C*) M13α-NBA RNase S′.

It is clear that a temperature-dependent model simpler than the third-order Gibbs polynomial model (T^3 model) cannot be used at low temperature and has been found to be unacceptable at room temperature; therefore it would be reasonable to apply a more complex model in the intervening temperature region only if the facts demand a more complex fit. In fact, the facts do not require a different function; the model as described has been found to have both strong correlative power and very good predictive power.

The fitted thermal data ($\Delta G°(T)$, $\Delta H°(T)$, $\Delta W°(T)$, $T\Delta S°(T)$, and $\Delta Cp°(T)$) are reasonable not only over the measured experimental range (near room temperature) but in the low-temperature limit. The fitting curves do nothing strange in the experimentally inaccessible region, but rather smoothly approach the low-temperature limit. The thermal dependency presented here is the best (and essentially the only) approach known.

Computational procedure for protein unfolding

It has been known for some time that the folded states of many proteins are only marginally stable under the best of conditions. The delicate balance between folded and unfolded forms may be disrupted by such environmental changes as an increase in temperature, variation of pH, and addition of denaturants or Hofmeister anions. In the case of protein unfolding or DNA unwinding, however, the nonlinear (T^3) model for

$$\Delta H(T) = \alpha + \beta T^3 e^{\gamma T} \tag{6}$$

must be applied (Chun, 1994, 1997b, 1998, 1999).

In this treatment, the Gibbs free energy data, as shown in Fig. 6, were fitted to a three-term nonlinear enthalpy polynomial function in the 295–330 K temperature range, the range in which experiments have been conducted. Once evaluated as shown in Fig. 6, the coefficients α, β, and γ were fitted to other thermodynamic parameters. $\Delta H(T)$, $\Delta G(T)$, $\Delta Cp(T)$, $T\Delta S(T)$, and $\Delta W(T)$ are defined as follows:

$$\Delta G(T) = \alpha - \beta T e^{\gamma T}(\gamma T - 1)/\gamma^2 \tag{7}$$

$$\Delta Cp(T) = \beta T^2 e^{\gamma T}(\gamma T + 3) \tag{8}$$

$$T\Delta S(T) = \beta T e^{\gamma T}[T^2 + (\gamma T - 1)/\gamma^2] \tag{9}$$

$$\Delta W(T) = -\beta T e^{\gamma T}(\gamma T - 1)/\gamma^2 \tag{10}$$

Values of these thermodynamic parameters were regenerated from the fitted coefficients α, β, and γ, using the general nonlinear model procedure of IMSL's statistical subroutines as shown in Fig. 7, *A–D*. Each data point between 0 and 350 K was evaluated with extrapolation of F-statistics (Chun, 1991; Barr et al., 1985). $F = 0.001$; thus the goodness of fit of experimental data was 99.8% or better in each case.

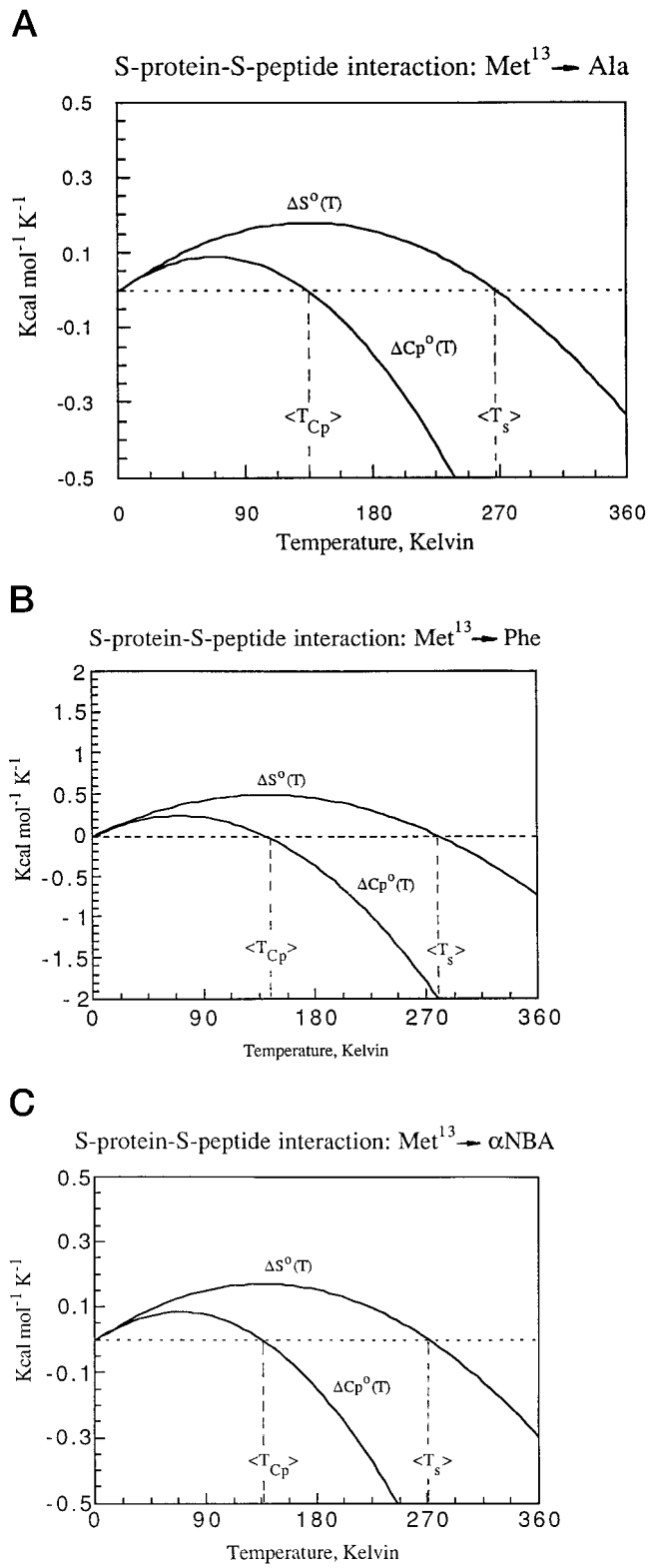

FIGURE 5 A close-up view of a portion of the fragment complementation reaction of S-protein of RNase S′ with substitutions at Met^{13} of S-peptide, over the temperature range of 0 K to 360 K, with the magnitude of the *y*-axis reduced to 0.5 to −0.5 (except for *B*, where it is 5 to −5) kcal mol^{-1} K^{-1}. Thermodynamic molecular switch occurs when $\Delta Cp°(T)$ at $\langle T_{Cp}\rangle$ changes sign from positive to negative, while $\Delta S°(T)$ or $T\Delta S°(T)$ changes from positive to negative at $\langle T_s\rangle$, as shown in Tables 2 and 4. (*A*) M13A RNase S′: Both $\Delta Cp°(T)$ and $\Delta S°(T)$ or $T\Delta S°(T)$ reach a maximum at 65 K and 135 K, respectively. (*B*) M13F RNase S′: Both $\Delta Cp°(T)$ and $\Delta S°(T)$ or $T\Delta S°(T)$ reach a maximum at 65 K and 135 K, respectively. (*C*) M13α-NBA RNase S′: Both $\Delta Cp°(T)$ and $\Delta S°(T)$ or $T\Delta S°(T)$ reach a maximum at 70 K and 135 K, respectively.

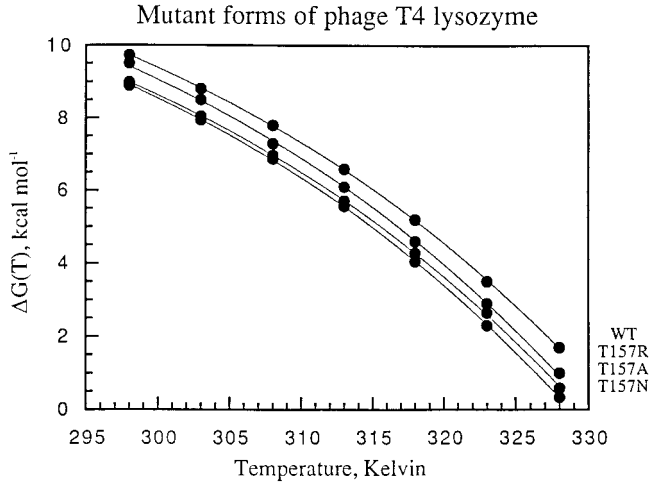

FIGURE 6 Thermodynamic plots of the Gibbs free energy change of the mutant form of phage T4 lysozyme as a function of temperature in the temperature range of 298–328 K, at pH 2.5 in 20 mM KH_2PO_4 containing 25 mM KCl and 0.5 mM dithiothreitol, based on data reported by Connelly et al. (1991). The experimental data for differential scanning calorimetric measurements were evaluated with the nonlinear model data reported by Connelly et al. (1991). The experimental data for differential scanning calorimetric measurements were evaluated with the nonlinear model procedure of statistical analysis of the IMSL subroutine (Chun, 1991; Barr et al., 1985). The solid line represents fitted data $F = 0.01$; thus the goodness of fit of the experimental data was 98.8% or better in each case. $\Delta H(T) = \alpha + \beta T^3 e^{\gamma T}$. The expansion coefficients α, β, and γ for wild-type T157, T157R, T157A, and T157N were as follows:
WTT157: $\alpha = 15.09 \pm 0.35$ kcal mol^{-1}; $\beta = 1.44 \times 10^{-9}$ kcal mol^{-1} K^{-3}; $\gamma = 2.37 \times 10^{-2}$ kcal mol^{-1} K^{-1}.
T157R: $\alpha = 15.12 \pm 0.65$ kcal mol^{-1}; $\beta = 1.06 \times 10^{-9}$ kcal mol^{-1} K^{-3}; $\gamma = 2.37 \times 10^{-2}$ kcal mol^{-1} K^{-1}.
T157A: $\alpha = 14.48 \pm 0.34$ kcal mol^{-1}; $\beta = 1.38 \times 10^{-9}$ kcal mol^{-1} K^{-3}; $\gamma = 2.35 \times 10^{-2}$ kcal mol^{-1} K^{-1}.
T157N: $\alpha = 14.49 \pm 0.24$ kcal mol^{-1}; $\beta = 1.33 \times 10^{-9}$ kcal mol^{-1} K^{-3}; $\gamma = 2.41 \times 10^{-2}$ kcal mol^{-1} K^{-1}.
WT157: $R^2 = 0.998$; chisig = 0.0057; PR > $F = 0.01$
T157R: $R^2 = 0.998$; chisig = 0.0189; PR > $F = 0.01$
T157A: $R^2 = 0.999$; chisig = 0.0055; PR > $F = 0.01$
T157N: $R^2 = 0.999$; chisig = 0.0057; PR > $F = 0.01$.

RESULTS AND DISCUSSION

Gibbs free energy change as a function of temperature

Plots of the Gibbs free energy change as a function of temperature of ribunuclease S′ and S-peptides–S-protein interaction shown in Figs. 1 and 2, *A–C* are typical of biological reactions in that they show a Gibbs free energy

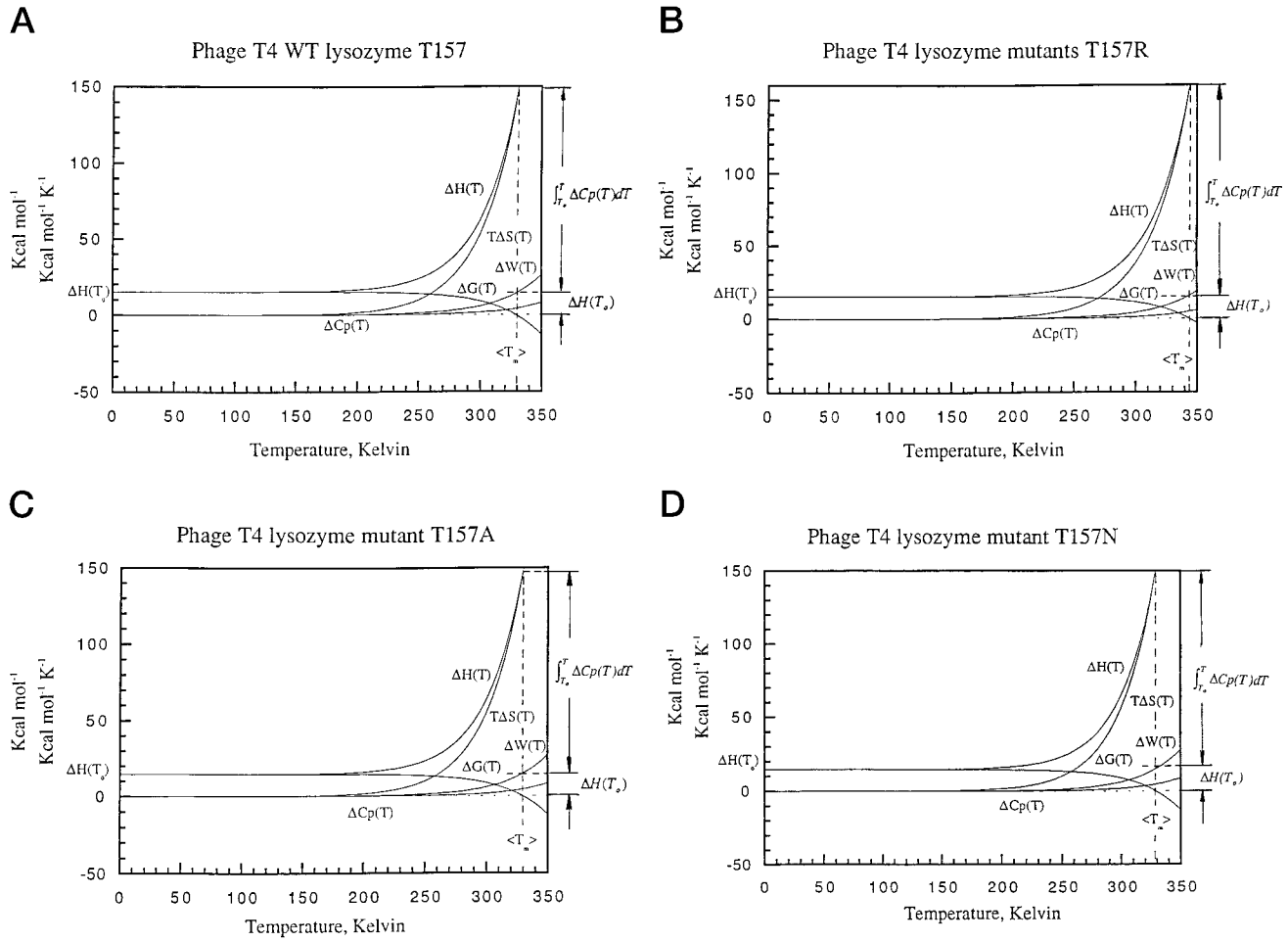

FIGURE 7 Thermodynamic plot of the reversible unfolding of wild-type T4 phage lysozyme in which the threonine residue at position has been replaced by four different residues. The experimental conditions were identical to those in Fig. 6. (*A*) T157: $\Delta H(T) = \Delta H(T_0) + \int_0^T \Delta Cp(T)\mathrm{d}T = 15.09 + 149.93 = 165.02$ kcal mol^{-1}. (*B*) T157R: $\Delta H(T) = \Delta H(T_0) + \int_0^T \Delta Cp(T)\mathrm{d}T = 15.12 + 162.13 = 177.25$ kcal mol^{-1}. (*C*) T157A: $\Delta H(T) = \Delta H(T_0) + \int_0^T \Delta Cp(T)\mathrm{d}T = 14.48 + 147.76 = 162.24$ kcal mol^{-1}. (*D*) T157N: $\Delta H(T) = \Delta H(T_0) + \int_0^T \Delta Cp(T)\mathrm{d}T = 14.49 + 151.78 = 166.27$ kcal mol^{-1}.

change minimum at the point of equilibrium in the system at $\langle T_s \rangle$, the stable temperature at which $T\Delta S°(T) = 0$. This is not the case, however, for the S-protein–S-peptide reaction in which norleucine has been substituted for Met[13] (shown in Fig. 2 *D*). The fitted coefficients of α, β, and γ were found to be -33.52 ± 0.78 kcal mol^{-1}, 6.997×10^{-4} kcal mol^{-1} K^{-2}, and -1.403×10^{-6} kcal mol^{-1} K^{-3}, respectively. No values for $\langle T_h \rangle$, $\langle T_s \rangle$, or $\langle T_m \rangle$ could determined. In this case, it is inferred that no fragment complementation reaction takes place, that is, the S-peptide does not realign with the ribonuclease S′ system to form the native structure, although it may bind elsewhere as part of a transfer reaction.

Analysis of the Planck-Benzinger thermal work function

Thermodynamic plots of ribonuclease S′ fragment complementation reactions (Figs. 3, *A* and *B*, and 4, *A–C*) are shown as a function of temperature. The innate temperature-invariant enthalpy, $\Delta H°(T_0)$, may be evaluated at four points on these curves: $\langle T_h \rangle$, $\langle T_s \rangle$, $\langle T_m \rangle$, and zero Kelvin (Chun, 1994, 1995, 1996a,b, 1997a, 1998) (see Table 1).

A plot of the Gibbs polynomial function, $\Delta G°(T) = \alpha + \beta T^2 + \gamma T^3$, as a function of temperature exhibits an initial value of zero for $\Delta G°(T)$ at $\langle T_h \rangle$ and a negative value of maximum magnitude for $\Delta G°(T)$ at $\langle T_s \rangle$, and the $\Delta G°(T)$ value again reaches zero at $\langle T_m \rangle$. Here $\langle T_s \rangle$ is the stable temperature at which $T\Delta S°(T) = 0$, $\langle T_m \rangle$ is the melting temperature, and $\langle T_h \rangle$ is the harmonious temperature at which $\Delta G°(T)$ is zero, $\Delta C°_p(T)$ approaches zero, and $T\Delta S°(T)$ reaches a positive maximum. Values of the innate temperature-invariant enthalpy at $\langle T_h \rangle$, $\langle T_s \rangle$, $\langle T_m \rangle$ are compared with those at zero K as shown in Figs. 3, *A* and *B*, and 4, *A–C* (see Table 1).

1. $\Delta H°(T_0) = \Delta W°(T_h)$, $\Delta G°(T) = 0$ at $\langle T_h \rangle$.

TABLE 1 Comparison of $\Delta H(T_0)$ at $\langle T_h \rangle$, $\langle T_s \rangle$, $\langle T_m \rangle$ and zero K for the S-protein–S-peptide interaction with various substitutions of Met[13]

Substitution	$\Delta H^\circ(T_0)$ at $\langle T_h \rangle$ (kcal mol^{-1})	$\Delta H^\circ(T_0)$ at $\langle T_s \rangle$ (kcal mol^{-1})	$\Delta H^\circ(T_0)$ at $\langle T_m \rangle$ (kcal mol^{-1})	$\Delta H^\circ(T_0)$ at 0 K (kcal mol^{-1})	$\langle T_h \rangle$	$\langle T_s \rangle$ (K)	$\langle T_m \rangle$	$\int \Delta Cp^\circ dT$
M13A RNase S′	25.67 ± 0.63	24.46 ± 0.39	24.21 ± 0.57	25.05 ± 0.57	185	270	340	−85.00
M13F RNase S′	85.69 ± 0.35	85.39 ± 0.13	85.39 ± 0.38	85.55 ± 0.38	225	280	325	−105.83
M13α-NBA RNase S′	22.32 ± 0.19	22.33 ± 2.32	22.01 ± 0.57	23.06 ± 0.57	186	270	350	−82.03
M13 norleucine RNase S′	—	—	—	−33.52 ± 0.13	—	—	—	—
20S RNase S′ 20-S[I]	34.05 ± 1.30	34.05 ± 0.54	34.02 ± 0.36	34.05 ± 1.30	180	273	345	−118.25
13S RNase S′ 13-S[II]	19.49 ± 1.00	19.49 ± 0.42	19.01 ± 0.13	19.49 ± 1.10	160	265	345	−88.62

Compiled using the general linear model procedure of statistical analysis of IMSL subroutine. $F = 0.001$; thus the goodness of fit of the experimental data was 99.8% or better in each use. Observed values for $\langle T_m \rangle$ are consistent with experimental values. (Reprinted from P. W. Chun, 1997a, *J. Phys. Chem.* 101B:7835. ©1997, American Chemical Society.)

2. $\Delta H^\circ(T_0) = \Delta W^\circ(T_s)_{max} + \Delta G^\circ(T_s)_{min}$, $\Delta H^\circ(T_S) = \Delta G^\circ(T_S)_{min}$ at $\langle T_s \rangle$.
3. $\Delta H^\circ(T_0) = \Delta W^\circ(T_m)$, $\Delta G^\circ(T) = 0$ at $\langle T_m \rangle$, and one can define the heat of reaction as $\Delta H^\circ(T) = \Delta H^\circ(T_0) + \int_{T_0}^{T} \Delta C_p^\circ(T) dT$.
4. $\Delta H^\circ(T_0)$ is evaluated at zero K.

The values of $\Delta W^\circ(T)$ and $\Delta G^\circ(T)$ exhibit a positive maximum and negative value of maximum magnitude (negative minimum), respectively, at $\langle T_s \rangle$; therefore, the innate temperature-invariant enthalpy, $\Delta H^\circ(T_0) = \Delta W^\circ(T_s)_{max} + \Delta G^\circ(T_s)_{min}$ at $\langle T_s \rangle$. The innate temperature-invariant enthalpy at the melting temperature is, by the integrated Kirchhoff expression, $\Delta H^\circ(T) = \Delta H^\circ(T_0) + \int_0^T \Delta Cp^\circ(T) dT$, where $\Delta H^\circ(T)$ and $T\Delta S^\circ(T)$ are of the same magnitude, $\Delta W^\circ(T) = \Delta H^\circ(T_0)$, and $\Delta G^\circ(T)$ approaches zero. The nature of the biochemical thermodynamic compensation that takes place between $\langle T_h \rangle$ and $\langle T_m \rangle$ may be characterized by evaluating $\Delta H^\circ(T_0)$ and the heat capacity integrals, as shown in Figs. 3, *A* and *B*, and 4 *A–C*, and Table 1.

We found values for the innate temperature-invariant enthalpy, $\Delta H^\circ(T_0)$, for the fragment complementation reaction of 20S-RNase S′ and 13S-RNase S′ in conformational transition at neutral pH to be 34.05 ± 1.30 and 19.49 ± 1.10 kcal mol^{-1}, respectively. The innate temperature-invariant enthalpy for 20S[I] is nearly the same as those for RNase A in 20% or 30% glycerol (Chun, 1996a), which are 37 and 33 kcal mol^{-1}, respectively. This we infer to involve the dimeric form of both RNase A and 20-S[I]. The $\Delta H^\circ(T_0)$ value for 13-S[II] is ~19 kcal mol^{-1}, which we infer to involve the monomeric form. Our results are consistent with findings reported by Crestfield et al. (1962).

Each of three $\Delta H^\circ(T_0)$ values of M13F RNase S′ complementation reactions are three to four times greater than those of M13A or M13 α-NBA, as shown in Table 1. This suggests that with substitution of phenylalanine-13 strong site-specific interaction prevails, resulting in dimer or higher aggregates.

Burley and Petsko (1985), in analyzing neighboring aromatic groups in four biphenyl peptides or peptide analogs and 34 proteins, reported that 60% of aromatic side chains in proteins are involved in aromatic pairs, 80% of which form networks of three or more interacting aromatic side chains. In phenyl ring centroids, the dihydral angles approached 90°.

A possible model for the fragment complementation reaction of S-peptide with S-protein if phenylalanine were to

TABLE 2 Thermodynamic molecular switch in $\Delta Cp^\circ(T)$ and $\Delta S^\circ(T)$

Fragment-complementation reaction	Thermodynamic quantities	Molecular switch at temperature, Kelvin	Effect on sign of $\Delta G^\circ(T)$ or $\Delta H^\circ(T)$
20S RNase S′	$\Delta Cp^\circ(T) = 0$ at $\langle T_{Cp} \rangle$	135	(+) → (−)
	$\Delta S^\circ(T) = 0$ at $\langle T_s \rangle$	270	(+) → (−)
13S RNase S′	$\Delta Cp^\circ(T) = 0$ at $\langle T_{Cp} \rangle$	133	(+) → (−)
	$\Delta S^\circ(T) = 0$ at $\langle T_s \rangle$	265	(+) → (−)
M13F RNase S′	$\Delta Cp^\circ(T) = 0$ at $\langle T_{Cp} \rangle$	135	(+) → (−)
	$\Delta S^\circ(T) = 0$ at $\langle T_s \rangle$	280	(+) → (−)
M13A-RNase S′	$\Delta Cp^\circ(T) = 0$ at $\langle T_{Cp} \rangle$	130	(+) → (−)
	$\Delta S^\circ(T) = 0$ at $\langle T_s \rangle$	275	(+) → (−)
M13αNBA-RNase S′	$\Delta Cp^\circ(T) = 0$ at $\langle T_{Cp} \rangle$	135	(+) → (−)
	$\Delta S^\circ(T) = 0$ at $\langle T_s \rangle$	270	(+) → (−)
Norleucine 13-RNase S′	$\Delta Cp^\circ(T) = 0$ at $\langle T_{Cp} \rangle$	—	—
	$\Delta S^\circ(T) = 0$ at $\langle T_s \rangle$	—	—

be substituted for Met^{13} incorporates feasible strong, site-specific Phe-Phe interaction ($Phe^{8} \rightarrow Phe^{120}$ and $Phe^{13} \rightarrow Phe^{8} \rightarrow Phe^{120}$), giving rise to the large innate temperature-invariant enthalpy (Chun, 1997a).

Thermodynamic molecular switch in biological systems

$\Delta H^{\circ}(T)$ and $\Delta S^{\circ}(T)$ are simple fundamental thermodynamic functions. In each case the respective value at a given temperature is determined in a straightforward way, using the following expression:

$$[\Delta H^{\circ}_{T} - \Delta H^{\circ}(T_0)] = \int_0^T \Delta Cp^{\circ}(T),$$

$$\text{whereas } \Delta S^{\circ}_{T} = \int_0^T (\Delta Cp^{\circ}/T)\mathrm{d}T$$

Note that the value of the enthalpy change at 0 K, $\Delta H^{\circ}(T_0)$, is important, but distinct and separate from the thermal agitation term. Many authors have entirely ignored this when dealing with biological systems.

In contrast, the Gibbs free energy function is a composite quantity, defined as a trade-off of $\Delta H^{\circ}(T)$ and $\Delta S^{\circ}(T)$ terms:

$$\Delta G^{\circ}(T) = \Delta H^{\circ}(T) - T\Delta S^{\circ}(T)$$

$$\Delta G^{\circ}(T) = \Delta H^{\circ}(T_0) + \int_0^T \Delta Cp^{\circ}\mathrm{d}T - T\int_0^T (\Delta Cp^{\circ}/T)\mathrm{d}T$$

In consequence $\Delta G^{\circ}(T)$ displays an interesting variety of behavior patterns as the temperature changes. ΔG° can change sign (K_{eq} from <1 to >1 or vice versa) only if ΔH° and ΔS° remain of the same sign. That is to say,

1. If ΔH° is (+) and ΔS° is (+) then ΔG° goes from (+), which is unfavorable, to (−), which is favorable (from $K_{eq} < 1$ to $K_{eq} > 1$).
2. If ΔH° is (−) and ΔS° is (−) then ΔG° goes from (−), which is favorable, to (+), which is unfavorable (from $K_{eq} > 1$ to $K_{eq} < 1$).

All chemical reactions can be characterized by four different thermodynamic behavior patterns, in the simplest case where the sign of $\Delta Cp^{\circ}(T)$ is fixed. These may be charted as in Table 3.

In biological systems, $\Delta H^{\circ}(T)$ and $\Delta S^{\circ}(T)$ are positive at low temperature. As the reaction temperature increases, both $\Delta H^{\circ}(T)$ and $\Delta S^{\circ}(T)$ become negative; that is scheme 4 of Table 3 goes to scheme 1: $\Delta H^{\circ}(+)$, $\Delta S^{\circ}(+) \rightarrow \Delta H^{\circ}(-)$, $\Delta S^{\circ}(-)$. In this thermodynamic switch unique to and characteristic of biological systems, $\Delta Cp^{\circ}(T)$ changes from positive to negative at $\langle T_{Cp}\rangle$, the temperature at which $\Delta Cp^{\circ}(T) = 0$, where $\Delta S^{\circ}(T)$ is at a positive maximum close to $\langle T_{Cp}\rangle$. As seen in Table 4, the negative Gibbs free energy minimum at $\langle T_s\rangle$ of 20S-RNase S′ and 13S-RNase S′ falls at 270 and 265 K, respectively. For M13A-RNase S′ and M13 α-NBA-RNase S′ values for $\Delta G^{\circ}(T)$ at $\langle T_s\rangle$ are 275 and 270 K. In M13F-RNase S′, the value of $\Delta G^{\circ}(T_s)_{min}$ at $\langle T_s\rangle$ is highest, 280 K. The sign of $\Delta S^{\circ}(T)$ or $T\Delta S^{\circ}(T)$ changes from positive to negative at $\langle T_s\rangle$, as shown in Table 4:

$$\Delta G^{\circ}(T)(+) \rightarrow \Delta G^{\circ}(T_s)_{min}(-) \rightarrow \Delta G^{\circ}(T)(+)$$

$$\Delta S^{\circ}(+) \rightarrow \Delta S^{\circ}(-), \quad \text{where } \Delta S^{\circ} = 0 \text{at} \langle T_S\rangle$$

At temperature $\langle T_S\rangle$, a simple algebraic sum of $\Delta W^{\circ}(T_s)$ and $\Delta H^{\circ}(T_S)$ yields the value of $\Delta H^{\circ}(T_0)$, i.e., $\Delta H^{\circ}(T_0) = \Delta W^{\circ}(T_S)_{max} + \Delta H^{\circ}(T_S)$ (see Figs. 3, *A* and *B*, 4 *A*, and 5 *C*). At $\langle T_s\rangle$ there exists an optimal balance of $\Delta H^{\circ}(T_S) = \Delta G^{\circ}(T_S)_{min}$ and $T\Delta S^{\circ}(T)$, so there will be a minimum negative Gibbs free energy change and the maximum work can be accomplished.

In 13S-RNase S′ and 20S-RNase S′, $\Delta Cp^{\circ}(T)$ reaches a maximum at 65 K, while the sign changes from positive to negative at $\langle T_{Cp}\rangle = 135$ K, $\Delta Cp^{\circ}(T) = 0$. $\Delta Cp^{\circ}(T)(+) \rightarrow \Delta Cp^{\circ}(T)(-)$ at $\langle T_{Cp}\rangle$. $\Delta S^{\circ}(T)$ or $T\Delta S^{\circ}(T)$ in both cases is at a maximum at 130 K. In the mutants M13A-RNase S′, M13F-RNase S′, and M13α-NBA-RNase S′, $\Delta Cp^{\circ}(T)$ reaches a maximum at 65 K, 65 K, and 70 K, respectively, while $\Delta S^{\circ}(T)$ or $T\Delta S^{\circ}(T)$ for all three is at a maximum at 135 K.

This communication demonstrates that the critical factor is a temperature-dependent heat of reaction, $\Delta Cp^{\circ}(T)$, which is positive at low temperature but switches to a negative value at $\langle T_{Cp}\rangle$, the temperature at which $\Delta Cp^{\circ}(T) = 0$, as

TABLE 3 Thermodynamic behavior patterns of chemical reactions

Thermodynamic quantities	Standard Gibbs free energy change $\Delta G^{\circ}(T)$		Sign changes	Characteristic chemical reactions
	Low temperature	High temperature		
1. $\Delta H^{\circ}(-)$, $\Delta S^{\circ}(-)$	(−) favorable	(+) unfavorable	Yes	$NH_3 + HCl \longleftarrow NH_4Cl$
2. $\Delta H^{\circ}(-)$, $\Delta S^{\circ}(+)$	(−) favorable	(−) favorable	No	$I_2 + Cl_2 \Leftrightarrow 2ICl$
3. $\Delta H^{\circ}(+)$, $\Delta S^{\circ}(-)$	(+) unfavorable	(+) unfavorable	No	$2HCl \Leftrightarrow H_2 + Cl_2$
4. $\Delta H^{\circ}(+)$, $\Delta S^{\circ}(+)$	(+) unfavorable	(−) favorable	Yes	$PCl_5 \Leftrightarrow PCl_3 + Cl_2$

TABLE 4 Thermodynamic molecular switch in Gibbs free energy change

Fragment-complementation reaction	Thermodynamic quantities	Molecular switch at temperature, Kelvin	Effect on sign of $\Delta G^\circ(T)$ or $\Delta H^\circ(T)$
20S-RNase S′	$\Delta G^\circ(T)$ at $\langle T_h \rangle$	183	(+) → (−)
	$\Delta G^\circ(T)$ at $\langle T_s \rangle$	270	Negative minimum
	$\Delta G^\circ(T)$ at $\langle T_m \rangle$	345	(−) → (+)
	$\Delta H^\circ(T)$	258	(+) → (−)
13S-RNase S′	$\Delta G^\circ(T)$ at $\langle T_h \rangle$	165	(+) → (−)
	$\Delta G^\circ(T)$ at $\langle T_s \rangle$	265	Negative minimum
	$\Delta G^\circ(T)$ at $\langle T_m \rangle$	343	(−) → (+)
	$\Delta H^\circ(T)$	248	(+) → (−)
M13F-RNase S′	$\Delta G^\circ(T)$ at $\langle T_h \rangle$	225	(+) → (−)
	$\Delta G^\circ(T)$ at $\langle T_s \rangle$	280	Negative minimum
	$\Delta G^\circ(T)$ at $\langle T_m \rangle$	325	(−) → (+)
	$\Delta H^\circ(T)$	275	(+) → (−)
M13A-RNase S′	$\Delta G^\circ(T)$ at $\langle T_h \rangle$	183	(+) → (−)
	$\Delta G^\circ(T)$ at $\langle T_s \rangle$	275	Negative minimum
	$\Delta G^\circ(T)$ at $\langle T_m \rangle$	340	(−) → (+)
	$\Delta H(T)$	255	(+) → (−)
M13αNBA RNase S′	$\Delta G^\circ(T)$ at $\langle T_h \rangle$	178	(+) → (−)
	$\Delta G^\circ(T)$ at $\langle T_s \rangle$	270	Negative minimum
	$\Delta G^\circ(T)$ at $\langle T_m \rangle$	345	(−) → (+)
	$\Delta H^\circ(T)$	255	(+) → (−)
M13Norleucine RNase S′	—	—	—

shown in Figs. 3, *C* and *D*, and 5, *A–C*, and Table 2. Note that the switch in the sign of $\Delta Cp^\circ(T)$ causes the change in $\Delta G^\circ(T)$ and hence a change in the equilibrium constant, K_{eq}, and/or spontaneity.

In protein unfolding, such as the unfolding of wild-type T4 phage lysozyme in which the threonine residue at position 157 has been replaced by four different amino acid residues (Fig. 7), the process obviously differs over the full temperature range. In this case, there is a single cut-off point, $\langle T_m \rangle$, at which energy is unfavorable but entropy is favorable and $\Delta G = 0$, as shown in Fig. 7 (see Table 5). Note that the thermal agitation energy is 10 times greater than the innate temperature-invariant enthalpy. The difference in the innate temperature-invariant enthalpy between folded and unfolded forms is small, as little as 2–17 kcal mol^{-1} (Chun, 1994, 1998, 1999). There is no trade-off between $\Delta H(T)$ and $T\Delta S(T)$ in protein unfolding (Chun, 1994, 1997b, 1999), nor is any thermodynamic molecular switch observed, that is, $\Delta H(T)$, $T\Delta S(T)$ and $\Delta Cp(T)$ remain positive and $\Delta G(T)$ changes from positive to negative.

What is the importance of $\langle T_s \rangle$?

Here the balance of $\Delta H^\circ(T_S) = \Delta G^\circ(T_S)_{min}$ and $T\Delta S^\circ(T)$ demands in the system are favorable. At this temperature, there will be a minimum negative Gibbs free energy change, and the maximum work can be accomplished for biological processes such as transpiration, digestion, reproduction, or locomotion, among others. When $\Delta G^\circ(T) = 0$ for a biological system (or for that matter, any energy system), such work is impossible. The possibility of the existence of life processes is not a clear and urgent demand of the physical universe. In fact, life exists only over a limited temperature range when the balance of energy and entropy demands is favorable. There is a lower cutoff point, $\langle T_h \rangle$, where entropy is favorable but energy (enthalpy) is unfavorable, and an upper cutoff, $\langle T_m \rangle$, above which energy (enthalpy) is favorable but entropy is unfavorable. Only between these two limits where $\Delta G^\circ(T) = 0$ is the net chemical driving force (indicated by $\Delta G^\circ(T)$) favorable for such biological processes as protein folding, protein-protein interaction, or protein self-assembly. Within this temperature range there will be a point with minimum (negative) free energy change, $\langle T_s \rangle$, at which the maximum work can be done.

TABLE 5 Comparison of $\Delta H(T_0)$ at 0 K and $\langle T_m \rangle$ for the unfolding of phage T4 lysozyme in which the threonine residue at position 157 has been replaced by seven different residues

	$\Delta H(T_0)$ at 0 K (kcal mol^{-1})	$\Delta H(T_0)$ at $\langle T_m \rangle$ (kcal mol^{-1})	$\int \Delta Cp(T)dT$ $[\Delta H(T_m) \equiv T\Delta S(T_m)]$ (kcal mol^{-1})	$\langle T_m \rangle$ (K)
WT(T15)	15.09 ± 0.35	14.93 ± 0.17	146.77 ≅ 146.99	330
T157R	15.12 ± 0.65	15.15 ± 0.34	162.13 ≅ 162.88	345
T157A	14.48 ± 0.34	14.43 ± 0.22	147.76 ≅ 147.50	330
T157N	14.49 ± 0.24	14.46 ± 0.57	151.78 ≅ 152.37	330

The experimental data for differential scanning calorimetric measurements in 20 mM KH_2PO_4 containing 25 mM KCl and 0.5 mM KCl and 0.1 mM dithiothreitol adjusted with HCl to pH 2.5 were evaluated with the nonlinear model procedure of statistical analysis of the IMSL subroutine. Each data point between 0 and 350 K was evaluated with extrapolation of F-statistics (Chun, 1991; Barr et al., 1985).

The critical role of the temperature-dependent heat capacity change of reaction

The Gibbs free energy function may be expressed as

$$\Delta G^\circ(T) = \Delta H^\circ(T_0) + \int_0^T \Delta Cp^\circ \mathrm{d}T - T\int_0^T (\Delta Cp^\circ/T)\mathrm{d}T$$

In consequence $\Delta G^\circ(T)$ displays an interesting variety of behavior patterns as the temperature changes. Most typical processes of biological molecules or biopolymers, for example, protein folding or protein-protein interactions, show $\Delta H^\circ(T)$ positive (unfavorable) and a positive $\Delta S^\circ(T)$ (favorable) at low temperature, due to a positive $(\Delta Cp^\circ/T)$. If $\Delta Cp^\circ(T)$ were positive, and the sign invariant at all temperatures, then this combination would predict that $\Delta G^\circ(T)$ for an association equilibrium process would change from positive to negative as temperature increases. Because $\Delta G^\circ(T)$ for biological systems shows a complicated behavior, wherein $\Delta G^\circ(T)$ changes from positive to negative, then reaching a negative (and thus favorable) minimum, before finally becoming positive as temperature increases, it is clear that a deeper thermodynamic explanation is required.

This communication demonstrates that the critical factor is the temperature-dependent $\Delta Cp^\circ(T)$ (heat capacity change) of reaction, which is positive at low temperature but switches to a negative value at $\langle T_{Cp}\rangle$, well below the ambient range at which $\Delta Cp^\circ(T) = 0$.

$$\Delta Cp^\circ(T)(+) \rightarrow \Delta Cp^\circ(T)(-) \quad \text{at } \langle T_{Cp}\rangle$$

This thermodynamic switch determines the behavior patterns of the Gibbs free energy change and hence a change in the equilibrium constant, K_{eq}, and/or spontaneity. The subsequent, mathematically predictable changes in $\Delta H^\circ(T)$, $\Delta S^\circ(T)$, $\Delta W^\circ(T)$, and $\Delta G^\circ(T)$ give rise to classically observed behavior patterns in biological reactivity that may be seen in the ribonuclease S′ fragment complementation reactions. In cases of protein unfolding such as wild-type T4 phage lysozyme in which the threonine residue at position 157 has been replaced by four different amino acid residues, no thermodynamic molecular switch is observed.

The S-peptide–S-protein fragment complementation reaction provides a unique means of delineating the role of each amino acid substitution and studying the effects of substitution on the structure and folding dynamics of proteins. A detailed thermodynamic analysis such as we have described should be an essential component of any future studies involving the site-directed, mutagenic approach to the examination of structure-function problems in proteins.

APPENDIX I: THE GIAUQUE FUNCTION AND THE PLANCK-BENZINGER THERMAL WORK FUNCTION

It should be noted that the Gibbs free energy for an ideal diatomic molecule in the standard state is $G^\circ_{\text{effective}} = G^\circ_{\text{total}} - E^\circ_0 = -NkT \ln f/e$, where $G^\circ_{\text{effective}}$ is the thermodynamic potential. What counts are the effective minimum values at chemical equilibrium obtainable from the partition function, f (Moelwyn-Hughes, 1957). The Gibbs free energy function was subsequently defined by Giauque (Giauque, 1930a,b; Giauque and Blue, 1930; Giauque and Meads, 1941; Giauque and Kemp, 1938) as $(G^\circ_T - H^\circ_0)/T = -\psi/T$, then $(G^\circ_T - H^\circ_0) = -\psi^\circ = -\{T\int_0^T (Cp^\circ/T)\mathrm{d}T - \int_0^T (Cp^\circ \mathrm{d}T\}$ when expressed as the heat capacity term. Values for $-\psi^\circ$ for pure solids are tabulated in the National Bureau of Standards' Circular 500 (Chase et al., 1985) and JANAF thermodynamic tables (Rossini and Wagnman, 1952). For an ideal gas or pure solid, this function would be equivalent to the Planck-Benzinger thermal work function, $W^\circ(T) = H^\circ_0 - G^\circ(T)$ (Benzinger, 1971). The Giauque function, $G^\circ_T - H^\circ_0 = -\psi^\circ$, has been sterile so far in terms of its application to biological systems. In contrast, the equivalent (although independently derived) Planck-Benzinger thermal work function has been quite fruitful.

In 1971, T. H. Benzinger proposed a thermal work function to take into account both Boltzmann statistical energy effects and energies of quantum-mechanical bonds. While the latter are usually not altered significantly in micromolecular reactions, it was Benzinger's conjecture that the large-scale and long-range changes of conformation that accompany protein folding or assembly might generate significant energy differences because of the cumulative alteration of their numerous covalent bond structures. It is probably true that the Giauque function could have been used and would have been appropriate—it simply was not. Rather, Benzinger pursued a separate path, although one that can be related to the work of Giauque and other chemists.

Planck's characteristic function (Planck, 1927) may be expressed as $\psi = H/T - S$. A proper substitution of the Kirchhoff equation (Moelwyn-Hughes, 1957; Lewis and Randall, 1961), $\Delta H^\circ(T) = \Delta H^\circ(T_0) + \int_0^T \Delta Cp^\circ \mathrm{d}T$ and $\Delta S^\circ = \int_0^T (\Delta Cp^\circ/T)\mathrm{d}T$, into the above expression will yield

$$\frac{\Delta G^\circ}{T} = \frac{\Delta H^\circ(T_0)}{T} + \frac{1}{T}\int_0^T \Delta Cp^\circ \mathrm{d}T - \left[\int_0^T (\Delta Cp^\circ/T)\mathrm{d}T\right]$$

$$\Delta G^\circ = \Delta H^\circ(T_0) - \left[T\int_0^T (\Delta Cp^\circ/T)\mathrm{d}T - \int_0^T \Delta Cp^\circ \mathrm{d}T\right]$$

By Benzinger's definition: $\Delta W^\circ = [T\int_0^T (\Delta Cp^\circ/T)\mathrm{d}T - \int_0^T \Delta Cp^\circ \mathrm{d}T]$

$$\Delta G^\circ = \Delta H^\circ(T_0) - \Delta W^\circ$$

The Planck-Benzinger thermal work function is therefore expressed as

$$\Delta W^\circ(T) = \Delta H^\circ(T_0) - \Delta G^\circ(T)$$

The Planck-Benzinger thermal work function, $\Delta W^\circ(T)$, represents the strictly thermal components of any intra- or intermolecular bonding term in a system, that is, energy other than the inherent difference of the 0 K portion of the interaction energy term at absolute zero Kelvin.

APPENDIX II: MINIMUM REQUIREMENTS FOR CORRECT TREATMENT OF TEMPERATURE DEPENDENCE OF K_{eq} IN BIOLOGICAL SYSTEMS

A simplistic integration of $(\partial \ln K_{eq}/\partial T) = (\Delta H^\circ/RT^2)$ with the assumption that $\Delta H_T^\circ = \Delta^\circ_{Ref}H + \Delta Cp^\circ T$ has frequently been presented. However, there is absolutely no independent evidence for this "one-adjustable term" assumption in biological systems, that is, that $\Delta Cp^\circ(T)$ is fixed. The basic equations are correct, but realistically, $\Delta Cp^\circ(T)$ is an elaborate function of temperature.

Second, to define ΔG° as a function of temperature from enthalpy measurements,

$$\frac{\partial[(-RT\ln K_{eq})/T]}{\partial(1/T)} = \frac{\partial(\Delta G^\circ/T)}{\partial(1/T)} = \Delta H^\circ$$

$$[\Delta G^\circ(T)/T] = -\int_0^T [\Delta H^\circ(T)/T^2]\mathrm{d}T$$

Here again, the basic equations are correct, but ΔH°_T is a detailed function of temperature as controlled by $\Delta Cp^\circ(T)$.

By Kirchhoff's law, the heat of reaction consists of two terms, the innate temperature-invariant enthalpy and heat capacity integrals (thermal agitation energy):

$$\Delta H^\circ(T) = \Delta H^\circ(T_0) + \int_0^T \Delta Cp^\circ \mathrm{d}T$$

Again $\Delta Cp^\circ(T)$ is not a constant or even a two-term function of the type $(A + BT)$, but again depends on temperature in a detailed way:

$$\Delta G^\circ(T) = \Delta H^\circ(T) - T\Delta S^\circ(T)$$

$$\Delta G^\circ(T) = \Delta H^\circ(T_0) + \int_0^T \Delta Cp^\circ \mathrm{d}T - T\int_0^T (\Delta Cp^\circ/T)\mathrm{d}T$$

Note that two temperature-dependent terms are needed on the right, one to account for ΔH°_T as a function of temperature, and the second to account for the ΔS°_T variation with temperature. Thus, in the thermodynamic analysis of the Gibbs free energy change as a function for biological systems, a number of additional factors must be considered in the measurement of K_{eq}. In the case of $\Delta G^\circ(T)$ of formation, phase transition is indeed important. When dealing with $\Delta G^\circ(T)$ of reaction, no phase transition is taking place. In this case, all the thermodynamic functions are continuous.

The thermodynamic methods we use are based on an absolute requirement that all computed values must be in agreement with K_{eq} values (and therefore with values of $\Delta G^\circ(T)$ of reaction) of biological systems over a temperature range of 273 to 342 K. Consider a hypothetical reaction involving biological systems,

$$\Delta S^\circ_T(\text{reaction}) = \Delta S^\circ_T(\text{product}) - \Delta S^\circ_T(\text{reactant})$$

Our work does not in any way assume that ΔS°_T of a given species is zero, but rather that the limit of ΔS°_T of reaction approaches zero at 0 K. The methods we have used do not give any access to ΔG°_T, ΔH°_T, or ΔS°_T of a species, but only to ΔG°_T, ΔH°_T, or ΔS°_T of a particular biological reaction.

There is no basis an any case to compare a value for $\Delta G^\circ(T)$ of formation of a small molecule with $\Delta G^\circ(T)$ of reaction, even one involving that molecule. Comparing $\Delta G^\circ(T)$ of formation of a small molecule with $\Delta G^\circ(T)$ of reaction of a system of large biological molecules is meaningless. Note particularly that Johari (1997), in dealing with evaluation of temperature-invariant enthalpy, computed values only for ΔS°_0 of formation of a particular (small molecule) species. This does not directly relate to a ΔS°_T of biological reaction.

I thank Dr. Robert J. Hanrahan, Department of Chemistry, University of Florida, for his enlightening discussion and suggestions. This work was supported by a faculty development award, Division of Sponsored Research, University of Florida.

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