# Contribution of Translational and Rotational Motions to Molecular Association in Aqueous Solution

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ABSTRACT Much uncertainty and controversy exist regarding the estimation of the enthalpy, entropy, and free energy of overall translational and rotational motions of solute molecules in aqueous solutions, quantities that are crucial to the understanding of molecular association/recognition processes and structure-based drug design. A critique of the literature on this topic is given that leads to a classification of the various views. The major stumbling block to experimentally determining the translational/rotational enthalpy and entropy is the elimination of vibrational perturbations from the measured effects. A solution to this problem, based on a combination of energy equi-partition and enthalpy-entropy compensation, is proposed and subjected to verification. This method is then applied to analyze experimental data on the dissociation/unfolding of dimeric proteins. For one translational/rotational unit at 1 M standard state in aqueous solution, the results for enthalpy ($H^o_{tr}$), entropy ($S^o_{tr}$), and free energy ($G^o_{tr}$) are $H^o_{tr} = 4.5 \pm 1.5RT$, $S^o_{tr} = 5 \pm 4R$, and $G^o_{tr} = 0 \pm 5RT$. Therefore, the overall translational and rotational motions make negligible contribution to binding affinity (free energy) in aqueous solutions at 1 M standard state.

## GLOSSARY

$\rho$ = $N/V$, number density
$M$ = molecular weight
$\det(\mathbf{A})$ = determinant of the inertial tensor $\mathbf{A}$
$C$ = concentration in molarity; in particular, $C^0 = 1$ M
$\gamma_j$ = stoichiometric coefficient of the $j$th molecular species in a chemical reaction
$R$ = gas constant (4.184 J $K^{-1}$ $mol^{-1}$)
$S^o$ = standard molar or partial molar entropy of a molecular species
$S^o_g$ and $S^o_l$ = $S^o$ in the gas phase and in the liquid phase, respectively
$\Delta S^o$ = standard entropy change of a chemical reaction
$\Delta S^o_{solvation}$ = solvation entropy
$S^o_t$, $S^o_r$, and $S^o_{tr}$ = translational, rotational, and translational + rotational entropy, respectively
$\Delta S^o_{vap}$ = vaporization entropy
$\Delta S^o_{noncovalent}$ = entropic effect of noncovalent perturbations by cross-linking two polypeptide chains, either folded or unfolded
$\Delta H^o$ = standard enthalpy change of a chemical reaction
$H^o_{tr}$ = translational + rotational enthalpy
$\Delta H^o_{noncovalent}$ = enthalpic effect of noncovalent perturbations caused by cross-linking two polypeptide chains, either folded or unfolded
$E^o_{tr}$ = translational + rotational energy

## INTRODUCTION

Most biochemical reactions involve molecular association/dissociation, from the relatively simple oligomerization reactions, in which only one type of molecule is involved, to the more complicated binding/recognition processes (e.g., drug-receptor interaction, enzyme-substrate association, and protein-DNA binding), in which at least two types of molecules are involved. Most reactions of this type result in a change in the number of molecules. For example, in a reaction where one complex dissociates into two subunits ($A_2 \leftrightharpoons A + A$, or $AB \leftrightharpoons A + B$), the number of molecules increases by one. Examples of reactions that conserve the number of molecules are the folding/unfolding of monomeric proteins ($N \leftrightharpoons U$) or a phosphorylation reaction of a substrate ($S + ATP \leftrightharpoons S\text{-}P + ADP$). Generally speaking, chemical reactions that lead to a change in the number of molecules can be characterized by a parameter $\nu$, defined as $\nu = \Sigma\gamma_j \neq 0$, with $\gamma_j$ being the stoichiometric coefficients with the stipulation that those of the products be positive and those of the reactants negative. For the dissociation of a dimer, $\nu = 1$.

For reactions of the type with $\nu \neq 0$, a unique contribution to the equilibrium constant arises from the overall translational/rotational motions of the molecule in terms of enthalpy and entropy, which could be significant depending on the concentrations of the molecules involved. Such a contribution arises because each molecule is an independent kinetic unit and as a result of the reaction, $6\nu$ degrees of overall motions, among which $3\nu$ are translational and $3\nu$ rotational, are converted either into or from, depending on the sign of $\nu$, $6\nu$ degrees of vibrational motions. We

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designate the enthalpy and entropy of each translational/rotational unit as $H^o_{tr}$ and $S^o_{tr}$, respectively, and hence the enthalpy and entropy change in a chemical reaction ($v \neq 0$) as $\Delta H^o_{tr}$ and $\Delta S^o_{tr}$, with the subscript *tr* referring to translational + rotational and the superscript *o* to standard state of specified concentration. Experimentally determined equilibrium constants or binding affinity always contain the $\Delta H^o_{tr}$ and $\Delta S^o_{tr}$ terms, in addition to contributions from noncovalent interactions. Indeed, this translational/rotational entropy effect has been used to explain the chelate effect (Calvin and Bailes, 1946; Westheimer and Ingraham, 1956) and the much smaller enthalpy requirement of intramolecular hydrogen bond formation compared with the intermolecular one (Jaffe, 1957). In the context of biochemical reactions, it has been pointed out years ago that $\Delta S^o_{tr}$ makes an important contribution to enzymatic catalysis (Page and Jencks, 1971; Page, 1977; Jencks, 1986). It must be emphasized that it is a mistake to equate $\Delta H^o_{tr}$ ($\Delta S^o_{tr}$) with the entire association enthalpy (entropy), which also includes many other terms, such as vibrational and solvation enthalpy (entropy) (Kollman, 1993; Gilson et al., 1997; Brady and Sharp, 1997a). On the other hand, any attempt to calculate binding affinity must take $\Delta H^o_{tr}$ and $\Delta S^o_{tr}$ into consideration and specify the concentration at which the calculations are made.

The evaluation of $\Delta H^o_{tr}$, $\Delta S^o_{tr}$, and $\Delta G^o_{tr}$ for molecular association reactions in aqueous solutions is the focus of this article. Through analysis of experimental results from our laboratories, we are able to give a narrow range for $H^o_{tr}$, $S^o_{tr}$, and $G^o_{tr}$ for each translational/rotational unit which in turn can be used to calculate $\Delta H^o_{tr}$, $\Delta S^o_{tr}$, and $\Delta G^o_{tr}$ in an association/dissociation reaction of any order (dimerization, trimerization, tetramerization, etc.). In this work, we do not discuss the issue of whether the residual motions of partners in a macromolecular complex should be treated as translational/rotational modes or as vibrational modes. Both treatments require a correct account of $H^o_{tr}$, $S^o_{tr}$, and $G^o_{tr}$ for each translational/rotational unit (a molecule or a complex) in the liquid phase.

This article is organized as follows. The first part is an overview of general statistical mechanical relationships on $H^o_{tr}$ and $S^o_{tr}$, which serves to clarify the question that we are attempting to answer. The second part is a critique of the results in the literature to illustrate the various viewpoints that exist and the origin of their differences. The third part is a presentation of our own results.

## GENERAL STATISTICAL MECHANICAL RELATIONSHIPS

Consider a multi-component system of $m$ molecular species at equilibrium with fixed temperature $T$ and pressure $P$. The number of molecules of each species is $N_j$ ($j = 1, 2, \ldots, m$) and the total number of molecules is $N$, i.e., $N = \Sigma N_j$. The classical isothermal-isobaric partition function, $\Theta$, for this system is (Hill, 1986):

$$\Theta = \int_0^\infty \frac{Z_N}{\left(\prod_{j=1}^{m} N_j! \Lambda_j^{3N_j}\right)} \cdot \exp(-PV/kT) d(PV/kT), \quad (1)$$

where $V$ represents volume, $\Lambda_j = h/\sqrt{2\pi M_j kT}$, with $M_j$ being the molecular weight of the $j$th species, $h$ Planck's constant, and $k$ the Boltzmann constant. $Z_N$ is the configurational integral, given as:

$$Z_N = \int \exp[-U(r)/kT] dr, \quad (2)$$

with $r$ representing the atomic coordinates and $U(r)$ the energy potential. From the partition function, the system's enthalpy and entropy can be obtained as (unless otherwise specified, all thermodynamic quantities are in molar units):

$$H^o = RT^2 \times \left[\frac{\partial \ln \Theta}{\partial T}\right]_{P,N} \quad (3)$$

$$S^o = RT \times \left[\frac{\partial \ln \Theta}{\partial T}\right]_{P,N} + R \ln \Theta, \quad (4)$$

where $R$ is the gas constant. The above formal relationships are valid for any system with fixed $T$ and $P$. However, except for a few simple model systems such as the ideal gas, it is not possible to obtain the configurational integral analytically. The next section gives the results for ideal gas systems to illustrate some of the basic concepts on this topic.

### $H^o_{tr}$ and $S^o_{tr}$ for ideal gas systems

For ideal gases, the configurational integral $Z_N$ can be obtained analytically in exact form, which gives the translational and rotational partition function for any one of the $m$ molecular species as (Hill, 1986):

$$\Theta_{tr} = \Theta_t \times \Theta_r = \left[\frac{kT}{P\Lambda_j^3}\right]^N \times \left[\frac{\pi^{1/2}}{\sigma} \times \left(\frac{8\pi^2 kT}{h^2}\right)^{3/2} \times \det(\mathbf{A}_j)^{1/2}\right]^N \quad (5)$$

The first part of Eq. 5 is the translational partition function, and the second part the rotational partition function. $\sigma$ is the symmetry factor ($\sigma = 1$ if there is no symmetry). For nucleic acids and monomeric proteins, $\sigma = 1$. $\det(\mathbf{A}_j)$ is the determinant of the inertial tensor, $\mathbf{A}_j$, of the $j$th molecular species. Then, from Eq. 5, the translational and rotational enthalpy and entropy of the $j$th molecular species can be

obtained (neglecting the temperature dependence of $\det(\mathbf{A}_j)$):

$$H^o_{tr} = 4RT \tag{6}$$

$$S^o_{tr} = S^o_t + S^o_r = \left[2.5R - R\ln\frac{N_j}{V}\Lambda_j^3\right] + \left[1.5R + R\ln\pi^{1/2}\left(\frac{8\pi^2 kT}{h^2}\right)^{3/2}\det(\mathbf{A}_j)^{1/2}\right] \tag{7}$$

Eqs. 6 and 7 give the translational/rotational enthalpy and entropy, respectively, for each molecular species. For a reaction involving several molecular species, the translational/rotational enthalpy and entropy change, $\Delta H^o_{tr}$ and $\Delta S^o_{tr}$, can be easily calculated from $H^o_{tr}$ and $S^o_{tr}$ of each molecular species.

From Eq. 6, it is clear that $H^o_{tr}$ depends only on the temperature. In contrast, $S^o_{tr}$ is much more complex.

### The translational entropy

The first part of Eq. 7, also called the Sackur-Tetrode equation, is the translational entropy, $S^o_t$. $S^o_t$ depends on molecular weight, through $\Lambda^3$, as $1.5R\ln M$ and concentration $\rho_o$ as $R\ln\rho_o$ ($\rho_o = N/V$). In systems of ideal gas, ideal solution, or ideally dilute solution, $S^o_t$ is the only one among the standard enthalpy and entropy functions that has concentration dependency, and it is for this reason that for reactions with $\nu \neq 0$, the standard concentration must always be specified. It is worth pointing out that the standard concentration for any solution must always be finite rather than infinitely dilute. At infinite dilution ($N/V \to 0$), $S^o_t$ diverges to infinity. The conventional standard concentration in biochemistry is 1 M (1 mol $L^{-1}$).

### Rotational entropy

The second part of Eq. 7 is the rotational entropy $S^o_r$. Unlike $S^o_t$, which is concentration dependent but is indifferent to the structure of the molecule, $S^o_r$ has no concentration dependency but depends on molecular structure through the term $\det(\mathbf{A})$. For folded proteins, $\det(\mathbf{A})$ can be readily calculated if the structure is known and is expected to have little temperature dependence. For unfolded proteins, $\det(\mathbf{A})$ is replaced by the ensemble average $\langle\det(\mathbf{A})\rangle$. The calculation of $\langle\det(\mathbf{A})\rangle$ requires the generation of an ensemble of unfolded conformations which is by no means trivial. Studies on $\langle\det(\mathbf{A})\rangle$, using abstract models, indicate that for random hard sphere chains $\ln\langle\det(\mathbf{A})\rangle$ scales linearly with the logarithm of chain length with the critical exponent $\eta$ ranging from 6 to 7, depending on the radii of the hard spheres (Yu and Wang, 1999; Wang and Yu, unpublished results). Such scaling behavior of $\langle\det(\mathbf{A})\rangle$ means that, for random homopolymers, $S^o_r$ depends on chain length $N$ as $0.5\eta R\ln N$. Consequently, $S^o_r$ depends on molecular mass as $0.5\eta R\ln M$ as chain length is proportional to molecular mass for homopolymers. Thus, for random homopolymers, both $S^o_t$ and $S^o_r$ are linear functions of $R\ln M$. For real unfolded proteins or other random polymers, the picture is more complicated. One thing can be certain, however, is that $\langle\det(\mathbf{A})\rangle$ of the unfolded state is larger than $\det(\mathbf{A})$ of the folded state because the unfolded state is more expanded (in fact, $\det(\mathbf{A})$ provides a measure of how expanded a molecule is). Thus, $S^o_r$ makes a contribution to the unfolding entropy even in the case of monomeric unfolding. The magnitude of this contribution is hard to estimate, although it is not expected to be large.

Perhaps the most notable feature of the results from ideal gas statistics on $H^o_{tr}$ and $S^o_{tr}$ is that they are completely uncorrelated. At any given temperature, $H^o_{tr}$ is a constant $4RT$, but $S^o_{tr}$ can take any value, depending on the volume $V$, because an ideal gas system can isothermally expand to any volume (of course, the density must be high enough to have a stable pressure). For a typical protein with a molecular weight in the range of 5–25 kDa at 1 M standard state and ambient temperature, both $S^o_t$ and $S^o_r$ are $\sim 25R$, with a total of $50R$ for $S^o_{tr}$. Thus, $TS^o_{tr} \approx 50RT$. Hence, at 1 M standard state, $T\,S^o_{tr}$(ideal gas) is an order of magnitude larger than $H^o_{tr}$(ideal gas) ($= 4RT$) with a difference on the order of $45RT$.

## EVALUATION OF $S^o_{tr}$ IN AQUEOUS SOLUTIONS IN THE LITERATURE

For the liquid state, the configurational integral, $Z_N$, cannot be obtained analytically. Therefore, $H^o_{tr}$ and $S^o_{tr}$ of a solute have to be evaluated either empirically or computationally. Over the years, different views and approaches have been taken to estimate $S^o_{tr}$, generating both excitement and controversy.

One approach states that as far as the translational and rotational motions are concerned, a liquid is no different from an ideal gas (Steinberg and Scheraga, 1963; Ben-Naim, 1978; Finkelstein and Janin, 1989; Erickson, 1989; Tidor and Karplus, 1994; Janin, 1995; Brady and Sharp, 1997a,b). Therefore, $S^o_{tr}$(liquid) = $S^o_{tr}$(ideal gas), and Eq. 7 is used to estimate $S^o_{tr}$(liquid). The essence of this approach is to treat the solvent as a structureless continuum (Prue, 1969). In this kind of approach, the effect of solvent is allocated entirely to a term called solvation with no explicit reference to the restrictions imposed on the translational/rotational motions of the solute molecule by the solvent.

A second approach is to correct $S^o_{tr}$(ideal gas) by the entropy of vaporization. This method has been used to obtain the corrections for small organic molecules; i.e., $S^o_{tr}$(liquid) = $S^o_{tr}$(ideal gas) $- \Delta S^o_{vap}$, where $\Delta S^o_{vap}$ is the vaporization entropy of the solute molecule after proper concentration and temperature correction (Page and Jencks, 1971; Page, 1977; Andrews et al., 1984; Doig and Williams,

1992). The conclusion is that the vaporization entropy correction on $S^o_{tr}$(ideal gas) for bimolecular dissociation reactions is very small ($<3R$) because the reactant has a higher molecular weight compared with the products and therefore higher boiling point and higher vaporization entropy. A variation of this approach is to use the sublimation entropy, $\Delta S^o_{sub}$, rather than $S^o_{tr}$(ideal gas) as the base value for correction. The correction term again is the vaporization entropy; i.e., $S^o_{tr}(\text{liquid}) = \Delta S^o_{sub} - \Delta S^o_{vap}$ (Searle and Williams, 1992). The essence of this variation is counting the residual motions of each unit in the molecular complex as translational/rotational rather than vibrational. For proteins and nucleic acids, the problem with this kind of approach is that their vaporization entropy from aqueous solutions is simply unknown and is certainly not expected to obey Trouton's rule, which states that, at the normal boiling temperature (i.e., $P = 1$ atm.), the vaporization entropy of non-associating liquids is $\sim 11R$ (Nash, 1984). Nonetheless, this is a step in the right direction compared with the first approach, which applies ideal gas statistics to macromolecules in aqueous solutions.

A third approach is to compare experimental standard dissociation entropy with empirical, structure-based entropic scoring functions and attribute the difference to $S^o_{tr}$. In one such estimate (Bohm, 1994) it was concluded, based on a data set of 45 protein-ligand complexes, the optimum value for $S^o_{tr}$ is $2.2R$. In another study (Murphy et al., 1994), it was found that the cratic entropy (for the definition of cratic entropy, see Gurney, 1953), with a value of $4R$ for aqueous solution, provides the best estimate for the loss of translational entropy (also see Adamson, 1954). Later, this conclusion was extended to include also the rotational entropy (Gomez and Freire, 1995). It is remarkable that two different scoring functions based on two different data sets give essentially the same numerical result for $S^o_{tr}$ as the numerical result relies crucially on the accuracy of the empirical scores assigned to other entropic terms, which are often much larger than the resultant $S^o_{tr}$.

A fourth approach is a force-field-based numerical evaluation of the configurational integral. The formalism for such an approach has been outlined by Gilson and coworkers (Gilson et al., 1997). The conclusion is that solvent-mediated interaction potential has to be included in calculating $S^o_{tr}$(liquid). The corollary of this conclusion is that $S^o_{tr}$(ideal gas) is not a good approximation for $S^o_{tr}$(liquid). In principle, such an approach of numerical evaluation of the configurational integral can be exact and, if carried out, is expected to give accurate values for $S^o_{tr}$. The challenge is twofold. First is to find a computationally tractable and yet accurate force field for macromolecular aqueous solutions. Second, the system should be large enough so that the result is valid in the thermodynamic limit ($N\rightarrow\infty$, $V\rightarrow\infty$, $N/V$ constant). This is an important point that is not always heeded to in molecular simulations. It is encouraging to see that calculation along this line has been carried out for benzene upon binding to a T4 lysozyme mutant (Hermans and Wang, 1997). In this calculation, 5668 water molecules are explicitly incorporated in the simulation, and the result obtained for $S^o_{tr}$ is $\sim 11.5R$ at 300 K close to Trouton's value for vaporization entropy. A variation of this approach, which evaluates the configurational integral not by force-field-based numerical computation, but using an argument based on the free volume theory of the liquid state, was given by Amzel (1997). In this variation, the volume term ($V$) in Eq. 7 is replaced with a term called the free volume ($V_f$), which is "the effective volume in which a particular molecule in the liquid can move and obey the perfect gas law" (Eyring and Hirschfelder, 1937). It deals with $S^o_t$ only, and the result obtained by Amzel is $5.3R$.

Occasionally, a combination of some of the above approaches is used (Novoty et al., 1989; Spolar and Record, 1994).

## Relationship among the various approaches

Denote the reduction of $S^o_{tr}$(ideal gas) caused by the restrictions of overall translational/rotational motions of a molecule imposed by the liquid phase as $\delta S^o_{tr}$; i.e., $\delta S^o_{tr} = S^o_{tr}(\text{liquid}) - S^o_{tr}(\text{ideal gas})$. The sharpest distinction among these approaches is the relationship between $\delta S^o_{tr}$ and the solvation entropy. The solvation entropy, $\Delta S^o_{solvation}$, refers to the entropy effect of transferring a motionless solute molecule from the gas phase to the liquid phase (Ben-Naim, 1978; Ben-Naim and Marcus, 1984). Although never explicitly stated, the first two approaches discussed above are diametrically opposed to each other in the following sense. The first approach allocates the vaporization entropy entirely to the solvation entropy (after proper adjustments of $T$, $P$, and $\rho$). In contrast, the second approach allocates the entire vaporization entropy to $\delta S^o_{tr}$. In this latter approach, the solvation entropy of a solute molecule devoid of overall translational/rotational motions would be zero. Therefore, in these two approaches, $\delta S^o_{tr}$ and $\Delta S^o_{solvation}$ are mutually exclusive. The third and the fourth approaches treat solvation and restriction of translational/rotational motions in a separate and complementary manner. Denote the entropy of a molecule in the gas and liquid phases (same $T$, $P$, and $\rho$) as $S^o_g$ and $S^o_l$, respectively, and let $\delta S^o = S^o_l - S^o_g$. Of course, we do not mean that at fixed $T$, $P$, and $\rho$, there can be two phases, one gas and one liquid. Such a statement violates the phase rule. Here, $S^o_g$ is simply the calculated molar entropy of the molecule using ideal gas statistics, and $S^o_l$ is the actual partial molar entropy of the molecule in the liquid state. Barring any conformational changes upon condensation/dissolution, then the four different approaches can be summarized as follows: $\delta S^o = \Delta S^o_{solvation}$, $\delta S^o_{tr} = 0$ (first approach); $\delta S^o = \delta S^o_{tr}$, $\Delta S^o_{solvation} = 0$ (second approach); $\delta S^o = \delta S^o_{tr} + \Delta S^o_{solvation}$ (third and fourth). In this regard, an interesting proposition proposed by Wertz (1980) is that $\delta S^o$

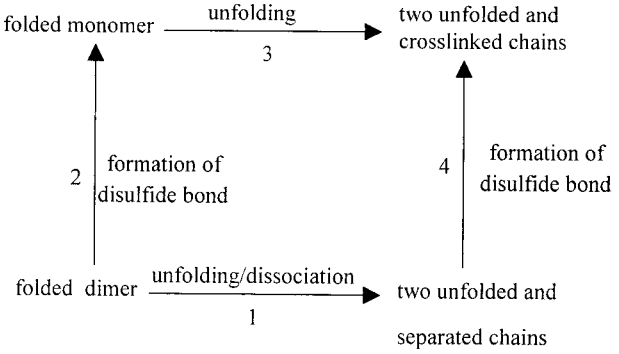


FIGURE 1 Thermodynamic cycle used to extract $S^o_{tr}$ from temperature-induced unfolding/dissociation reactions: step 1, unfolding/dissociation of the dimmer; step 2, cross-linking the two helices in the dimer, resulting in a disulfide-bridged monomer; step 3, unfolding of the disulfide-bridged monomer; step 4, cross-linking the two unfolded polypeptide chains. $\Delta S^o_1 + \Delta S^o_4 = \Delta S^o_2 + \Delta S^o_3$. $\Delta S^o_1 = \Delta S^o_{dimer}$ and $\Delta S^o_3 = \Delta S^o_{monomer}$ are the unfolding entropy of the dimer and monomer, respectively. $\Delta S^o_2 = \Delta S^o_{S\text{-}Sbond} + \Delta S^o_{noncovalent,folded}$ with $\Delta S^o_{S\text{-}Sbond}$ being the entropy of forming the S-S bond whereas $\Delta S^o_{noncovalent,folded}$ the entropic effect of all the noncovalent interactions caused by cross-linking the folded state, such as alteration of the vibrational modes. $\Delta S^o_4$ contains similar terms plus an extra $-S^o_{tr}$ term, due to the loss of a translational/rotational unit caused by the cross-link; i.e., $\Delta S^o_4 = \Delta S^o_{S\text{-}Sbond} + \Delta S^o_{noncovalent,unfolded} - S^o_{tr}$. The cross-linking entropy, $\delta\Delta S^0$, is given by $\delta\Delta S^o = \Delta S^o_{dimer} - \Delta S^o_{monomer} = \Delta S^o_1 - \Delta S^o_3 = \Delta S^o_2 - \Delta S^o_4 = S^o_{tr} + \delta\Delta S^o_{noncovalent}$. Therefore, $\delta\Delta S^o = S^o_{tr} + \delta\Delta S^o_{noncovalent}$, which is Eq. 11 in the text. The enthalpic quantities satisfy identical relationships. There is no assumption about $\delta\Delta H^o_{noncovalent}$ or $\delta\Delta S^o_{noncovalent}$ being zero.

$= \delta S^o_{tr} = \Delta S^o_{solvation} = -\Delta S^o_{vap}$ after proper adjustments of $T$, $P$, and $\rho$.

## EXPERIMENTAL DETERMINATION OF $S^o_{tr}$ IN AQUEOUS SOLUTIONS

### Overall strategy

Unlike computational procedures, experiments measure $\Delta S^o_{tr}$ of a reaction rather than $S^o_{tr}$ of each molecular species. For a pair of reactions with $\delta\nu = 1$, $\delta\Delta S^o_{tr}$ is caused by one translational/rotational unit. Hence, $\delta\Delta S^o_{tr} = S^o_{tr}$. In the following discussion, we will simply use $H^o_{tr}$ and $S^o_{tr}$ instead of $\delta\Delta H^o_{tr}$ and $\delta\Delta S^o_{tr}$. Following the approach outlined by Page and Jencks (1971), we obtain $S^o_{tr}$ by comparing standard reaction entropy of a dimeric, intermolecular dissociation/unfolding reaction ($\nu = 1$) with that of its monomeric, intramolecular counterpart in which the two units are tethered together ($\nu = 0$). The differences in unfolding enthalpy and entropy are:

$$\delta\Delta H^o = \Delta H^o_{dimer} - \Delta H^o_{monomer} \quad (8)$$

$$\delta\Delta S^o = \Delta S^o_{dimer} - \Delta S^o_{monomer} \quad (9)$$

The corresponding thermodynamic cycle is shown in Fig. 1. $\delta\Delta H^o$ and $\delta\Delta S^o$ contain contributions from noncovalent interactions that include vibrational perturbations (the six new modes and alteration of existing modes (Tidor and Karplus, 1994)) and possible hydration effect. Any attempt to extract $S^o_{tr}$ out of $\delta\Delta S^o$ has to take these noncovalent perturbations into account. Because perturbation can happen to both the folded and the unfolded state, its magnitude cannot be reliably estimated using methods such as normal mode analysis because they require knowledge of the structure. This is the drawback of such an approach that couples dissociation with unfolding. This problem has been a subject of discussion (Karplus and Janin, 1999; Privalov and Tamura, 1999). Here, we provide a tentative solution to this problem, based on our previous work with further refinement and elaboration.

### Estimation of noncovalent perturbations

Formally, the noncovalent perturbation components of $\delta\Delta H^o$ and $\delta\Delta S^o$ can be defined, respectively, using the following equations:

$$\delta\Delta H^o = H^o_{tr} + \delta\Delta H^o_{noncovalent} \quad (10)$$

$$\delta\Delta S^o = S^o_{tr} + \delta\Delta S^o_{noncovalent} \quad (11)$$

Note that $\delta\Delta H^o_{noncovalent}$ and $\delta\Delta S^o_{noncovalent}$ are the difference of noncovalent perturbations between the folded and the unfolded states (Fig. 1). $\delta\Delta H°$ and $\delta\Delta S°$ are experimentally determined quantities.

The starting point is the estimation of $H^o_{tr}$. The energy part of $H^o_{tr}$, $E^o_{tr}$, can be estimated by the equi-partition theorem, which is valid for classical systems in both gaseous and condensed states (Wannier, 1987). This theorem states that, at thermal equilibrium, each degree of motion has an average kinetic energy of $RT/2$. For crystals, another term, up to $RT/2$, would be added for each degree of motion. This term originates from intermolecular interaction. For six degrees, it amounts to $3RT$ exactly for $E^o_{tr}$(ideal gas) and $6RT$ at most for $E^o_{tr}$(crystal). Because the liquid state is in between the gas state and the solid state, $E^o_{tr}$(liquid) should have a value somewhere in the range of $3RT$ and $6RT$, depending on the system. In condensed phase, $E^o_{tr} \approx H^o_{tr}$ because the $PV$ term of $H^o_{tr}$ is negligible. Thus, $3RT \leq H^o_{tr}(\text{liquid}) \leq 6RT$, with an uncertainty of $3RT$. In our previous work, the contribution of intermolecular potential energy was neglected, and $H^o_{tr}$ was estimated to be $3RT$ (Yu et al., 1999). $H^o_{tr}$ is then substituted in Eq. 10 to obtain $\delta\Delta H^o_{noncovalent}$.

The estimation of $\delta\Delta S^o_{vi}$ is based not on a rigorous theoretical ground like that of $H^o_{tr}$. Rather, it is based on the empirical compensatory relationship between enthalpy and entropy of weak intermolecular interactions, which states that $\delta\Delta H^o_{noncovalent} \approx T\delta\Delta S^o_{noncovalent}$. Enthalpy-entropy compensation is a widely observed phenomenon in binding, folding, and solvation processes (Gilli et al., 1994; Searle et al., 1995; Westwell et al., 1995; Gallicchio et al., 1998; Liu and Guo, 2001), and several theoretical justifications have

been given using different approaches (Dunitz, 1995; Qian, 1998; Liu and Guo, 2001). With the enthalpy-entropy compensation, $S^o_{tr}$ can be estimated from $\delta\Delta H^o$ and $\delta\Delta S^o$ as:

$$S^o_{tr} \approx \delta\Delta S^o - \delta\Delta S^o_{noncovalent} = \delta\Delta S^o - \frac{\delta\Delta H^o_{noncovalent}}{T}$$

$$= \delta\Delta S^o - \frac{\delta\Delta H^o - H^o_{tr}}{T} = \frac{H^o_{tr}}{T} - \frac{\delta\Delta G^o}{T} \quad (12)$$

The uncertainty in the estimation of $H^o_{tr}$, which is $3RT$, will cause an uncertainty in the estimation of $S^o_{tr}$, which will be $3R$. Another source of uncertainty in the estimation of $S^o_{tr}$ is the enthalpy-entropy compensation relationship because the compensation cannot be exact as it leads to $\delta\Delta G^o_{noncovalent} = 0$, which obviously is not true. Thus, the result obtained in this manner is necessarily an approximation, and the validity of such approximation should be subjected to verification.

### Experimental results for the determination of $S^o_{tr}$

Two protein systems were investigated. One is a natural globular protein, *Streptomyces* subtilisin inhibitor (SSI) whose two units, each of 113 residues, associate to form a homodimer upon folding (Tamura and Privalov, 1997). The other system is a synthetic peptide in which two identical chains, each of 36 residues, form a two-stranded $\alpha$-helical coiled-coil (Yu et al., 1999). In the SSI case, a mutant was made with Asp83 replaced by Cys to form a disulfide bond between two molecules, resulting in a disulfide-bridged monomer. In the coiled-coil case, the two $\alpha$-helices of the coiled-coil were cross-linked by replacing Ser3 with Cys for inter-chain disulfide bond formation, resulting in a disulfide-bridged monomer. In both studies, the unfolding/dissociation reaction was induced by elevated temperature with the heat capacity monitored by differential scanning calorimetry. The measurements were carried out over a wide concentration range, and as expected, the dimer unfolding exhibits concentration dependency whereas the monomer unfolding is concentration independent (Fig. 2). Tables 1–3 give the calorimetric data on the unfolding reactions of SSI at pH 6.0, SSI at pH 3.0, and the coiled-coil at pH 2.0, respectively. From these data, the cross-linking enthalpy, $\delta\Delta H^o$, and entropy, $T\delta\Delta S^o$, are obtained to give the cross-linking free energy, $\delta\Delta G^o$ (Table 4).

From $\delta\Delta G^o$ and $H^o_{tr}$ (the median value of $H^o_{tr}$, $4.5RT$, was used for the calculation), $S^o_{tr}$ is calculated using Eq. 12 (Table 4). The average result for $S^o_{tr}$ is $5 \pm 4$ $R$. This result is slightly different from what was given before ($2.5 \pm 4$ $R$ for the coiled-coil and $2.5 \pm 2$ $R$ for SSI) because in previous analysis, the value of $H^o_{tr}$ was taken to be $3RT$ rather than $4.5RT$. Also, in the analysis of SSI, the noncovalent perturbations were neglected because there was hardly any structural perturbation in the folded state. We see that the correction is indeed small ($<2R$) and is within the range of experimental error. The perturbations caused by the cross-link are likely to be in the unfolded state for SSI.

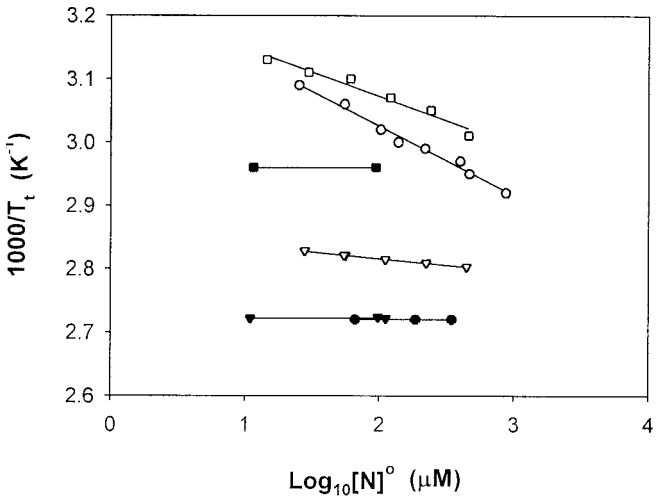


FIGURE 2 Concentration dependency of the transition temperature $T_t$ of protein complexes. $[N]^o$ is the total protein concentration in the dimer form in $\mu$M units. Solid symbols represent the cross-linked complex whereas open symbols represent the non-cross-linked complex with circles for the synthetic coiled-coil at pH 2.0, squares for *Streptomyces* subtilisin inhibitor (SSI) at pH 3.0, and triangles for SSI at pH 6.0. For a detailed discussion on the origin of the difference between pH 3.0 and pH 6.0 for SSI, see Tmura and Privalov (1997).

## DISCUSSION

### Validity of the results

For both SSI and the coiled-coil, the experimental $\delta\Delta H^o$ and $T\delta\Delta S^o$ are rather close at 1 M standard state, differing by less than $4RT$ in absolute value. This is in sharp contrast to the results of ideal gas statistics, which says that $TS^o_{tr}$ is an order of magnitude larger than $H^o_{tr}$ with their difference around $45RT$ for molecules in the range of 5–25 kDa. Of course, $\delta\Delta H^o$ and $T\delta\Delta S^o$ contain perturbations from noncovalent interactions. However, combining Eqs. 10 and 11, one obtains:

$$\delta\Delta H^o - T\delta\Delta S^o$$

$$= (H^o_{tr} - TS^o_{tr} + (\delta\Delta H^o_{noncovalent} - T\delta\Delta S^o_{noncovalent}) \quad (13)$$

Clearly, if $H^o_{tr}$ and $TS^o_{tr}$ were truly as different as predicted by ideal gas statistics, then the noncovalent perturbations, $\delta\Delta H^o_{noncovalent}$ and $T\delta\Delta S^o_{noncovalent}$, would have to be equally different in the opposite direction to make $\delta\Delta H^o$ and $T\delta\Delta S^o$ balanced. This is highly unlikely even if the compensatory relationship between $\delta\Delta H^o_{noncovalent}$ and $T\delta\Delta S^o_{noncovalent}$ is not perfect. Therefore, even without detailed analysis, the raw data indicate qualitatively that the ideal gas result on $S^o_{tr}$ cannot be applied to the liquid phase.

**TABLE 1 Calorimetric data for the unfolding of subtilisin inhibitor at pH 6.0**

| $[N]^0$ ($\mu$M) | $T_t$ (°C) | $\Delta H^0$ ($T_t$) | $\Delta S^0$ ($T_t$) | $\Delta H^{0*}$ (80°C) | $\Delta S^{0*}$ (80°C) |
|---|---|---|---|---|---|
| Cross-linked | | | | | |
| 113.4 | 94.0 | 831 | 2264 | 754 | 2029 |
| 97.7 | 94.2 | 828 | 2251 | 749 | 2017 |
| 11.0 | 94.2 | | | | |
| **Average** | | | | **751** | **2023** |
| Non-cross-linked | | | | | |
| 445 | 83.6 | 778 | 2125 | 748 | 2042 |
| 223 | 82.8 | 773 | 2113 | 750 | 2046 |
| 111 | 82.1 | 768 | 2096 | 750 | 2042 |
| 55.7 | 81.3 | 757 | 2067 | 746 | 2038 |
| 54.5 | 81.3 | 759 | 2071 | 748 | 2042 |
| 27.8 | 80.4 | 750 | 2046 | 746 | 2042 |
| **Average** | | | | **748** | **2042** |

$[N]^0$, total protein concentration in the dimer form; $T_t$, transition temperature; $\Delta H^0$ in kJ mol$^{-1}$; $\Delta S^0$ in J K$^{-1}$ mol$^{-1}$; standard concentration is 1 M.
*The experimental $\Delta H^0$ and $\Delta S^0$ are extrapolated to a common temperature for comparison. This common temperature (80°C in this case) is chosen to be roughly the mid-point of the transition temperature range to minimize error. The heat capacity change for this extrapolation is given by $\Delta C_P(T) = C_{PU}(T) - C_{PN}(T)$ with $C_{PU}(T) = A + BT + CT^2$ and $C_{PN}(T) = D + ET$. For subtilisin inhibitor, $A = 41.6$ kJ K$^{-1}$ mol$^{-1}$, $B = 0.248$ kJ K$^{-2}$ mol$^{-1}$, $C = -0.001314$ kJ K$^{-3}$ mol$^{-1}$, $D = 28.7$ kJ K$^{-1}$ mol$^{-1}$, $E = 0.202$ kJ K$^{-2}$ mol$^{-1}$ whereas $A = -3.8$ kJ K$^{-1}$ mol$^{-1}$, $B = 0.113$ kJ K$^{-2}$ mol$^{-1}$, $C = -0.0001374$ kJ K$^{-3}$ mol$^{-1}$, $D = 1.21$ kJ K$^{-1}$ mol$^{-1}$, $E = 0.045$ kJ K$^{-2}$ mol$^{-1}$ for the coiled-coil peptide.

The quantitative result rests on the accuracy of our procedure to eliminate $\delta\Delta S^o_{noncovalent}$ from $\Delta S^o$. This is answered by comparing the two different procedures afforded by the special case of the synthetic coiled-coil. In the coiled-coil system, the disulfide bond produced significant increase in helical structure as evidenced by the circular dichroism spectra of the two peptides: the non-cross-linked coiled-coil is 83% as helical as the cross-linked one. This increase in helicity is due to reduction of end fraying (Zhou et al., 1993). The extent of structural perturbation to the unfolded state is not known. Thus, in this system, it is mandatory to consider $\delta\Delta H^o_{noncovalent}$ and $\delta\Delta S^o_{noncovalent}$ for necessary corrections. Due to the sequential repetitiveness and structural regularity of the synthetic coiled-coil, there is an alternative way to correct for perturbations to the folded state caused by the cross-link. This is achieved by normalizing the unfolding enthalpy and entropy of the cross-linked peptide according to the helicity ratio of the two peptides. This method is completely independent of the general correction method based on energy equi-partition and enthalpy-entropy compensation and therefore provides a check for the validity of the general method.

The experimental $\delta\Delta H^o_{noncovalent}$ and $T\delta\Delta S^o_{noncovalent}$ for the coiled-coil are both $-4.9RT$, giving a $S^o_{tr}$ of $4.5R$ (Table 4). On the other hand, had perturbations to the folded state been corrected by helicity normalization, the resultant $\delta\Delta H^o$ and $T\delta\Delta S^o$ would be 7.4 and $6.5RT$, respectively. This leads to a $S^o_{tr}$ of $3.6RT$. Notice that although the normalization correction is rather significant such that $\delta\Delta H^o$ and $T\delta\Delta S^o$ changed their signs, the resultant $S^o_{tr}$ is hardly affected, with a difference of less than $1R$. This analysis demonstrates that two totally different procedures give the same result for $S^o_{tr}$ within $1R$, well within experimental error ($4R$). Thus, whether perturbations to the folded state are corrected alone first, using helicity normalization, or together with perturbations to

**TABLE 2 Calorimetric data for the unfolding of subtilisin inhibitor at pH 3.0**

| $[N]^0$ ($\mu$M) | $T_t$ (°C) | $\Delta H^{0*}$ ($T_t$) | $\Delta S^{0*}$ ($T_t$) | $\Delta H^0$ (60°C) | $\Delta S^0$ (60°C) |
|---|---|---|---|---|---|
| Cross-linked | | | | | |
| 92.4 | 65.1 | 548 | 1531 | 493 | 1356 |
| 11.6 | 65.1 | | | | |
| **Average** | | | | **493** | **1356** |
| Non-cross-linked | | | | | |
| 455 | 59.1 | 495 | 1347 | 505 | 1377 |
| 242 | 54.9 | 447 | 1218 | 505 | 1393 |
| 120 | 52.1 | 415 | 1109 | 508 | 1389 |
| 59.7 | 49.8 | 385 | 1017 | 505 | 1381 |
| 29.6 | 48.1 | 364 | 958 | 504 | 1385 |
| 14.6 | 46.3 | | | | |
| **Average** | | | | **505** | **1385** |

See Table 1 for definitions.
*The ionization heat of the histidine group in SSI has already been corrected (Tamura and Privalov, 1997).

**TABLE 3 Calorimetric data of the unfolding of the coiled-coil peptide at pH 2.0**

| $[N]^0$ ($\mu$M) | $T_t$ (°C) | $\Delta H^0$ ($T_t$) | $\Delta S^0$ ($T_t$) | $\Delta H^0$ (70°C) | $\Delta S^0$ (70°C) | $\Delta \hat{H}^o$* (70°C) | $\Delta \hat{S}^o$* (70°C) |
|---|---|---|---|---|---|---|---|
| Cross-linked | | | | | | | |
| 350 | 94.4 | 250 | 690 | 209 | 566 | 174 | 470 |
| 188 | 94.3 | 249 | 680 | 205 | 554 | 170 | 460 |
| 66 | 94.4 | | | | | | |
| **Average** | | | | **207** | **560** | **172** | **465** |
| Non-cross-linked | | | | | | | |
| 877 | 69.1 | 195 | 521 | 195 | 522 | | |
| 466 | 66.0 | 188 | 505 | 194 | 522 | | |
| 400 | 64.1 | 186 | 500 | 196 | 525 | | |
| 217 | 61.6 | | | | | | |
| 137 | 59.7 | | | | | | |
| 103 | 57.7 | | | | | | |
| 55 | 54.0 | | | | | | |
| 25 | 50.4 | | | | | | |
| | 60.0§ | 173 | 461 | 189 | 508 | | |
| **Average** | | | | **193** | **519** | | |

See Table 1 for definitions.

*$\Delta \hat{H}^0$ (= 0.83 $\Delta H^0$) and $\Delta \hat{S}^o$ (= 0.83 $\Delta S^0$) are the enthalpy and entropy values, respectively, of the cross-linked peptide normalized to the helicity of non-cross-linked one. This normalization procedure is based on the sequential repetitiveness of the coiled-coil peptide and serves as an independent check of our general approach to eliminate noncovalent perturbations from experimentally determined cross-linking enthalpy and entropy. See text for detail.

§Values obtained from the concentration dependence of $T_t$ (Yu et al., 1999).

the unfolded state, employing energy equi-partition and enthalpy-entropy compensation arguments, the result is the same. This provides strong evidence for the validity of our general method for correcting noncovalent perturbations.

For SSI at both pH 3.0 and 6.0, the cross-linking entropy is positive, indicating that the non-cross-linked dimer has a larger dissociation entropy than its cross-linked monomeric counterpart at 1 M standard state, i.e., $T\delta\Delta S^o$ is positive. On the other hand, for the coiled-coil, the dimer has lower dissociation entropy than the monomer at the same standard concentration, i.e., $T\delta\Delta S^o$ is negative (Table 4). However, this is not an anomaly on $S^o_{tr}$. Rather, the cross-linked coiled-coil has larger dissociation entropy because it is more helical than the non-cross-linked one and the larger dissociation entropy comes from the disruption of the extra helical segment. Once the helicity difference is removed through normalization, the dissociation entropy of the dimer becomes larger than the monomer, i.e., $T\delta\Delta S^o$ is positive. This example illustrates that dissociation/association entropy of a macromolecular complex has many attributes and should be interpreted with caution.

The value of $5 \pm 4R$ is about an order of magnitude lower than that estimated by ideal gas statistics ($50R$). Therefore, ideal gas statistics clearly is not applicable to macromolecular solutes in aqueous solution as far as translational/rotational motions are concerned. This conclusion, based on our experimental results, is in agreement with the theoretical approach outlined by Gilson et al. (1997), which concludes that intermolecular potential energy has to be included in the calculation of $S^o_{tr}$ (called external entropy in their work). The physical picture is that the translational/rotational motions of a molecule in the liquid phase are severely restricted. Indeed, it has been long established that greater restrictions on the rotation of a molecule in the liquid phase lead to larger entropy loss upon condensation (Everett, 1960).

**TABLE 4 Experimental cross-linking enthalpy, entropy, free energy, and resultant $H^o_{tr}$ and $S^o_{tr}$**

| System | Temperature (K) | $\delta\Delta H^0$ ($RT$) | $T\delta\Delta S^0$ ($RT$) | $\delta\Delta G^0$ ($RT$) | $H^o_{tr}$ ($RT$) | $S^o_{tr}$* ($R$) |
|---|---|---|---|---|---|---|
| SSI, pH 6.0 | 353.15 | −1.0 | 2.3 | −3.3 | 4.5 | 7.8 |
| SSI, pH 3.0 | 333.15 | 4.5 | 3.5 | 1.0 | 4.5 | 3.5 |
| Coiled-coil, pH 2.0† | 343.15 | −4.9 | −4.9 | 0.0 | 4.5 | 4.5 |
| Coiled-coil, pH 2.0‡ | 343.15 | 7.4 | 6.5 | 0.9 | 4.5 | 3.6 |
| **Average** | | | | | | **5 ± 4§** |

To facilitate comparison of results at different temperatures, all quantities are expressed in either $RT$ or $R$.

*$S^o_{tr}$ is calculated from $H^o_{tr}$ and $\delta\Delta G^0$ using Eq. 12. Here, the median value of $H^o_{tr}$ ($4.5RT$) is used with an uncertainty of $\pm 1.5RT$, which is smaller than the experimental error ($2RT$ for SSI and $4RT$ for the coiled-coil peptide).

†Value calculated using experimental $\Delta H^o_{dimer}$ and $\Delta S^o_{dimer}$.

‡Value calculated using helicity normalized $\Delta H^o_{dimer}$ and $\Delta S^o_{dimer}$, i.e., $0.83\Delta H^o_{dimer}$ and $0.83\Delta S^o_{dimer}$.

§The uncertainty range ($\pm 4R$) is taken to be the larger of the experimental errors of the two systems.

### Generality of the results

Our result on $S^o_{tr}$ is obtained by comparing the entropies of unfolded polypeptide chains (Fig. 1). How general is this result? The vast differences between these two proteins in both size (more than three times in terms of the number of amino acid residues) and shape (one globular and one filamentous) make it unlikely that this result is due to peculiarity of the systems employed. The value of $5 \pm 4R$ for $S^o_{tr}$ is in close agreement with the values obtained by empirical energetic scoring functions based on the folded protein structures, which is $2.2R$ in one case (Bohm, 1994) and $4R$ in another (Murphy et al., 1994), and $5 \pm 4R$ is also in accordance with the conclusion drawn by Bruice and co-workers (Bruice and Benkovic, 1964; Bruice and Lightstone, 1999) that in enzymic catalysis the average loss of translational/rotational entropy upon the formation of the transition state is $\sim$4.6 kcal mol$^{-1}$, equivalent to $7.5R$. A more significant agreement is afforded by the standard entropy $\Delta S^o$of the following reaction:

$$Ni(NH_3)_6^{2+} + 3ethylenediamine = Ni(ethylenediamine)_3^{2+} + 6NH_3,$$

which is $12R$ (Calvin and Bailes, 1946). The authors concluded that this entropy increase is mainly due to the fact that there are three more particles on the right side of the equation. Thus, for each translational/rotational unit, the entropy effect is $4R$, essentially the same as ours. Such agreement between totally different approaches and systems supports that our result is not confined to unfolded polypeptide chains but has general applicability to polar solutes in aqueous solutions. Deviation from this result is likely due to the shape rather the size of the solute molecule, analogous to the situation that within a homologous series it is mainly the molecular shape rather than the molecular weight that causes deviation from the Trouton-Hildebrande-Everret rule on vaporization entropy (Nash, 1984; Everett, 1960). Polarity of the solute molecule could be another source of deviation. Therefore, for macromolecules, our result should be most applicable to globular and unfolded proteins and is likely the upper limit of $S^o_{tr}$. In the case of long rod-shaped molecules like tropomyosin, greater restrictions might be imposed on its rotations and hence results in an even lower $S^o_{tr}$, much in the same way that normal hydrocarbons have greater entropy loss upon condensation than branched ones (Everett, 1960).

### Theoretical perspective

From a theoretical point of view, $5 \pm 4R$ is remarkably close to the cratic entropy, which is $4R$, and to the result obtained by Amzel (1997) based on the free volume theory, which is $5.3R$. However, such agreements should be viewed with caution. The cratic entropy, given by the formula $-R \ln x$, where $x$ is the mole fraction of the solute, is simply the ideal mixing entropy (Gurney, 1953). The practice of assigning the mole fraction $x$ of solutes at 1 M standard state a value of 1/55.5 and then equating $-R \ln x$ with $S^o_{tr}$ (or just $S^o_t$) is problematic from several accounts (Holtzer, 1994; Brady and Sharp, 1997a; Gilson et al., 1997). Amzel's analysis on the other hand, concerns only the translational part of $S^o_{tr}$. Furthermore, its numerical result rests upon the premise that in a 1 M aqueous solution, the free volume of a solute molecule ($V_{f,1M}$) and the free volume of a water molecule ($V_{f,w}$) in liquid water (concentration 55 M) satisfy the relationship: $V_{f,1M} = 55V_{f,w}$. The validity of this relationship for macromolecular solutes such as proteins certainly is questionable for two reasons. First, the huge volume of macromolecular solutes cannot be ignored. Consequently, the concentration of water at 1 M standard state is no longer 55 M as in the case for small molecule solutes like NaCl. This is despite the fictitious nature of the 1 M standard state (Yu, 2001). Second, even if the volume of the macromolecular solutes is ignored for a moment, then the macromolecular solute is still surrounded by 55 M rather than 1 M water molecules. Consequently, $V_{f,1M} \leq V_{f,w}$ rather than $V_{f,1M} = 55V_{f,w}$. However, Amzel's basic thesis, that the translational/rotational motions are restricted in the liquid state as compared with that in the gas state, is certainly valid and consistent with experimental results. Quantitatively, more refined theoretical analyses, such as numerical evaluation of the configurational integral, are needed to fully explain these experimental results. A criterion that any such computational approach should meet is the ability to reproduce numerically both the Trouton-Hildebrand-Everett rule for vaporization entropy and deviations from this rule (Nash, 1984; Everett, 1960).

### Contribution of overall translation/rotation to binding affinity

Our results demonstrate that, in an aqueous solution of 1 M, the overall translational/rotational motions make comparable and opposite contributions to bimolecular association enthalpy and entropy. The median value for the unfavorable entropic contribution is $5R$ at 1 M standard concentration, equivalent to $5RT$ in terms of Gibbs free energy. The median value for the favorable enthalpic contribution is $4.5RT$, independent of the concentration. Hence, the translational/rotational motions make negligible contribution to standard binding free energy, $\Delta G^o_{asso}$. Therefore, the entire standard binding affinity is due to contributions other than translational/rotational motions that form the intrinsic binding affinity. Put in mathematical terms,

$$\Delta G^o_{asso}(1\ M) = -G^o_{tr}(1\ M) + \Delta G_{intrinsic} \approx \Delta G_{intrinsic}, \text{ as } G^o_{tr}(1\ M) \approx 0. \quad (14)$$

It has to be emphasized that this conclusion is based solely on experimental results rather than any theoretical arguments. It is entirely empirical that the concentration at which $G^{o}_{tr} \approx 0$ happens to be around 1 M. No specific meaning has been or should be attached to the 1 M standard state. Furthermore, this conclusion applies only to association reactions in aqueous solution. It is certainly not valid for reactions in gas phase. Its applicability to reactions in other solvents might depend on the polarity of the solvents. However, because most biochemical association reactions happen in aqueous solutions, this conclusion has quite general significance. For instance, it explains why computational procedures that ignore the translational/rotational motions can still reproduce experimentally determined binding affinity (Murphy et al., 1993; Miyamoto and Kollman, 1993). Also, our result ($\Delta S^{o}_{tr} = 5 \pm 4R$ for dimer dissociation) is in agreement with the recent computer simulation result that activation entropy change for enzymic catalysis is much more limited than previously estimated (Villa et al., 2000). Of course, for a bimolecular complex at physiological concentrations, contribution from the translational/rotational motions to binding is not negligible because the translational part of $S^{o}_{tr}$ is concentration dependent. At arbitrary concentration $C$, another term $-R\mathrm{T} \ln C/C^{o}$ ($C^{o} = 1$ M) is added so that:

$$\Delta G_{asso}(C) = -RT \ln C/C^{o} + \Delta G_{intrinsic} \qquad (15)$$

## CONCLUSION

The contribution of translational/rotational motions to molecular association reactions in aqueous solution has been determined using a combination of experimental measurements and theoretical analysis. For one translational/rotational unit at 1 M standard state, the values for $H^{o}_{tr}$, $S^{o}_{tr}$, and $G^{o}_{tr}$ are as follows: $H^{o}_{tr} = 4.5 \pm 1.5RT$, $S^{o}_{tr} = 5 \pm 4R$, and $G^{o}_{tr} = 0 \pm 5RT$. For the dissociation of a complex made of $n$ subunits, the corresponding translational/rotational enthalpy, entropy, and free energy contributions are $(n - 1)H^{o}_{tr}$, $(n - 1)S^{o}_{tr}$, and $(n - 1)G^{o}_{tr}$.

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