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Molecular-size corrections to the strength of the hydrophobic effect: a critical review

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Abstract. Large increases in the strength of the hydrophobic effect and, consequently, in the estimates of the hydrophobic contribution to the thermodynamics of protein folding (and other biologically-relevant processes), have been recently advocated on the basis of the application, to model transfer thermodynamic data, of corrections for the solute/solvent size disparity. In this work we first examine the effect of molecular-size corrections on the values calculated from several types of model transfer data. For the transfer of a solute from an organic solvent to water, the above increase is exclusively associated with the application of a solute/water molecular-size correction. Secondly, we critically review and assess the several theoretical arguments that lead to these corrections. In particular, we show that, contrary to previous claims in the literature, the analysis of dissolution processes in terms of ideal-gas, intermediate states does not lead to the molecular-size correction term, but only to expressions equivalent (although not strictly identical) to those derived from the well-known Ben-Naim's statistical-mechanical approach. In general, the several analyses offered or discussed in this work disfavor the application of the solute/water molecular-size corrections.

Key words: Hydrophobic effect – Molecular-size corrections

Introduction

Quantitative information on the hydrophobic effect (for recent reviews, see Dill 1990; Creighton 1991; Sharp 1991; Blokzijl and Engberts 1993) is usually derived from the study of model processes in which small molecular weight substances are transferred from a non-aqueous environment (organic liquid, gas phase, ...) to aqueous solution. At least in principle, the transfer thermodynamic magnitudes (calculated, for instance, in a "number density scale") may be used to evaluate the hydrophobic contribution to protein folding (and other biologically relevant processes). It has recently been proposed that, prior to this kind of application, transfer Gibbs energies must be corrected for a contribution related to the disparity in molecular size between solute and solvent. Theoretical considerations (Sharp et al. 1991a, b; De Young and Dill 1990) suggest that the correction term is given by $RT \cdot (1 - r)$, where r is the solute to solvent molar volume ratio. [The sign of the correction term depends on whether it corresponds to the initial or the final state of the transfer process; in the latter case the correction term will carry a minus sign: $-RT \cdot (1 - r)$]. We will refer to terms of the $RT(1 - r)$ type as "molecular-size corrections".

Molecular-size corrections have non-trivial consequences for hydrophobicity scales and the calculated strength of the hydrophobic effect. For instance, upon correction, the value for the strength of the hydrophobic effect calculated from liquid hydrocarbon dissolution increases (Sharp et al. 1991a, b; De Young and Dill 1990) from about 100 J/mol per $Å^2$ of water accessible surface area (ASA) to about 200 $J \cdot mol^{-1} \cdot Å^{-2}$. Thus, it would appear that acceptance of the molecular-size corrections implies that the hydrophobic contribution to protein folding (and other biologically-relevant processes) has been grossly underestimated in many previous analysis. Several, more or less technical discussions on molecular-size corrections have appeared in recent literature (Sun et al. 1992; Holtzer 1992; Lazaridis and Paulaities 1992; Lazaridis and Paulaities 1993; Smith et al. 1993; Rashin 1993; Ben-Naim and Mazo 1993; Tuñon et al. 1993, 1994; Abraham and Sakellariou 1994; Giesen et al. 1994; Ben-Naim 1994; Lee 1994; Sitkoff et al. 1994; Simonson et al. 1994; Chan and Dill 1994) but, in spite of this, no general agreement appears to have been reached yet. As will be made clear below, the matter is rather complex because: a) for the transfer of a solute from an organic solvent to aqueous solution we must consider two different molecular-size corrections; b) these corrections may have different effects on values calculated from different types of model transfer data; c) molecular-size corrections have been previously derived from several (and dissimilar) theoretical arguments.

Clearly, the real issue of concern here is whether the current values for the strength of the hydrophobic effect (and, consequently, the estimates of the hydrophobic contribution to biologically relevant processes) must be revised upwards. With this issue in mind we will: 1) examine the effect of the two molecular-size corrections on the values calculated from several types of model transfer data; 2) critically review and assess the various theoretical arguments that lead to these corrections. In particular, and most important, we will show that, contrary to previous claims in the literature (Sharp et al. 1991b), the analysis of dissolution processes in terms of ideal-gas, intermediate states does *not* lead to the molecular-size correction term, but only to expressions equivalent (although not strictly identical) to those derived from Ben-Naim's statistical-mechanical approach (Ben-Naim 1978, 1993).

The effect of the molecular-size corrections on the values calculated from model transfer data

Consider the partitioning of a solute (molar volume $\bar{V}_2$) between an organic solvent (molar volume $\bar{V}_S$) and water (molar volume $\bar{V}_W$) and the two following molecular-size corrections: a) the correction for the size difference between the organic solvent and the solute, which is given by $RT \cdot (1 - \bar{V}_2/\bar{V}_S)$; b) the correction for the size difference between the solute and water, which is given by $-RT \cdot (1 - \bar{V}_2/\bar{V}_W)$. In principle, one may believe that both corrections should be applied, that neither of them should or, perhaps, that just one of them should. If no corrections are applied the transfer Gibbs energy (per mole of solute) would be given by:

$$\Delta\bar{G}_2\{S \rightarrow W\} \stackrel{e}{=} -RT \cdot \ln(\rho_2^W/\rho_2^S) \quad (1)$$

where ρ_2^W and ρ_2^S are the number densities of the solute in the aqueous and the organic solution respectively, and the symbol $\stackrel{e}{=}$ means that the relation only holds for the equilibrium number densities. According to Ben-Naim's approach (Ben-Naim1978, 1992) the above expression gives the Gibbs energy change (per mole of solute) associated with the transfer of a solute molecule from a fixed position in the organic solution to a fixed position in the aqueous solution (local transfer quantity). Application of the organic solvent/solute size correction leads to the following corrected transfer Gibbs energy:

$$\Delta\bar{G}_2'\{S \rightarrow W\} \stackrel{e}{=} -RT \cdot \ln(\rho_2^W/\rho_2^S) + RT \cdot (1 - \bar{V}_2/\bar{V}_S) \quad (2)$$

while upon application of both corrections we obtain:

$$\Delta\bar{G}_2''\{S \rightarrow W\} \quad (3)$$
$$\stackrel{e}{=} -RT \cdot \ln(\rho_2^W/\rho_2^S) + RT \cdot (1 - \bar{V}_2/\bar{V}_S) - RT \cdot (1 - \bar{V}_2/\bar{V}_W)$$

or,

$$\Delta\bar{G}_2''\{S \rightarrow W\} \quad (4)$$
$$\stackrel{e}{=} -RT \cdot \ln(\rho_2^W/\rho_2^S) - RT\bar{V}_2 \cdot (1/\bar{V}_S - 1/\bar{V}_W)$$

the latter equation being equivalent to Eq. (2) in De Young and Dill (1990) and identical to Eq. (11) in Sharp et al. (1991b).

We consider now the analysis of three different types of model transfer data sets (types A, B and C below):

Type A. We wish to investigate the effect of the organic solvent on the transfer thermodynamics. We then consider several transfer processes corresponding to the partitioning of a given solute (for instance, benzene) between several organic solvents (for instance, n-alkanes from octane to hexadecane) and water. This is the type of experimental data previously analyzed by De Young and Dill (1990). We do not expect the local environment of the benzene molecule in the organic solution to change drastically when the organic solvent chain length is increased from n=8 to n=16. In spite of this, the "uncorrected" transfer Gibbs energies calculated by using Eq. (1) show a significant dependence on the n value (see Fig. 1). As demonstrated by de Young and Dill (1990), however, this n-dependence is largely absent in the corrected transfer Gibbs energies calculated according to Eq. (4). The first point to note is that this effect is entirely due to the organic solvent/solute correction [that is, the n-dependence is already abolished in the corrected Gibbs energies calculated from Eq. (2); see Fig. 1]. The reason is obviously that the solute/water correction, $-RT \cdot (1 - \bar{V}_2/\bar{V}_W)$, is strictly constant in this case because we always deal with the same solute (the same $\bar{V}_2$ value; 89 cm^3/mol for benzene). On the other hand, the organic solvent/solute correction, $RT \cdot (1 - \bar{V}_2/\bar{V}_S)$, does change with the organic solvent (through the $\bar{V}_S$ value which ranges from 163 cm^3/mol for octane to 293 cm^3/mol for hexadecane) and cancels out the n-alkane chain-length dependence of the partition coefficient and, hence, that of the transfer Gibbs energy (Fig. 1). It is also apparent in Fig. 1 that the values of the transfer Gibbs energies for benzene almost double upon application of both molecular-size corrections (Eq. (4)). As a result, a value for the strength of the hydrophobic effect calculated by dividing the transfer Gibbs energies by the benzene ASA will also double upon application of both corrections (De Young and Dill 1990). Therefore, the second point to note is that the increase in Gibbs energy is mostly due to the solute/water correction (Fig. 1), which is much larger than the organic solvent/solute correction; the reason is, obviously, that the molar volume of water ($\bar{V}_W$ = 18 cm^3/mol) is much smaller than those of the n-alkanes employed as organic solvents.

Type B. It is not, of course, appropriate to calculate the value for the strength of the hydrophobic effect by simply dividing the transfer Gibbs energy of benzene by its ASA value. Firstly, aromatic rings show a partial hydrophilic character owing to their hydrogen-bond-acceptor character (Levitt and Perutz 1988; Suzuki et al. 1991; Rodham et al. 1993; Privalov and Makhatadze 1993; Makhatadze and Privalov 1994). Secondly, it is more reliable to analyze several transfer processes corresponding to the partitioning of several solutes of different size (a homologous series of substances, preferably) between a given organic solvent and water and then calculate the value for the strength of the hydrophobic effect as the slope of a plot of transfer Gibbs energy *versus* solute ASA (Sharp et al. 1991b; Hermann 1977). For illustration, we consider here

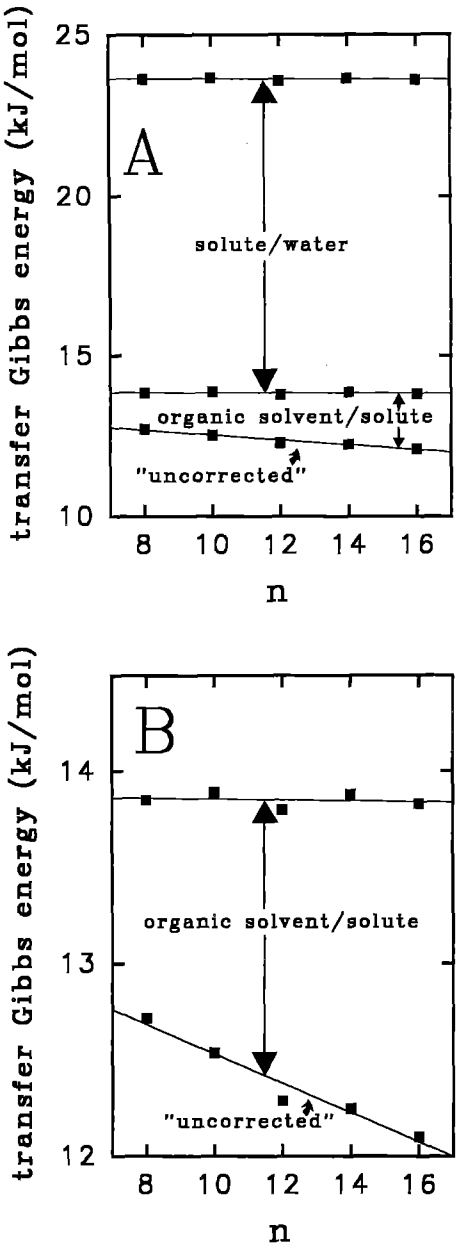

Fig. 1. **A** Effect of the application of the molecular-size corrections on the values for the Gibbs energy change associated with benzene transfer from several n-alkanes (n=8, 10, 12, 14, 16) to water. The "uncorrected" values were calculated from the K_V partition coefficients given by De Young and Dill (1990); the "corrected" values were derived form the uncorrected ones by use of Eqs (2) and (4). **B** Blowup showing that the application of the organic solvent/solute correction eliminates the n-dependence of the "uncorrected" values. Note, however, that only the application of the solute/water correction leads to a significant increase in the transfer Gibbs energies (see **A**)

the transfer of n-alkanes (n=1 to n=8) as solutes from n-octanol as organic solvent ($\bar{V}_S$=157 cm^3/mol) to water. (This is a hypothetical example; note, however, that partitioning of N-acetyl-amino-acid amides between n-octanol and water is the basis of one of the most widely employed hydrophobicity scales: Fauchere and Piska 1983). Now both $\bar{V}_S$ and $\bar{V}_W$ are constant while $\bar{V}_2$ varies, and the application of the molecular-size corrections will affect the calculated value for the strength of the hydrophobic effect, but only to the extent that these corrections change with the solute molar volume and, consequently, with the solute ASA. In fact, for the methane to octane set, there is an excellent linear relationship between molar volume and ASA:

$$\bar{V}_2 = \alpha + \beta \cdot \mathrm{ASA} \tag{5}$$

where $\alpha = -34.48$ cm^3/mol and $\beta = 0.534$ cm$^3 \cdot$ mol$^{-1} \cdot$ Å^{-2} (correlation coefficient R = 0.997), calculated from the liquid molar volumes given by Sharp et al. (1991b) and the ASA values from Giesen et al. (1994). Now, we can easily obtain expressions for the rate of change of the corrections with the solute ASA (values given below are for T = 25 °C):

organic solvent/solute correction,

$$\frac{\mathrm{d}}{\mathrm{dASA}}\{\mathrm{RT}\cdot(1-\bar{V}_2/\bar{V}_S)\} = -\mathrm{RT}\beta/\bar{V}_S \tag{6}$$
$$= -8\ \mathrm{J}\cdot\mathrm{mol}^{-1}\cdot\mathrm{Å}^{-2}$$

solute/water correction,

$$\frac{\mathrm{d}}{\mathrm{dASA}}\{-\mathrm{RT}\cdot(1-\bar{V}_2/\bar{V}_W)\} = \mathrm{RT}\beta/\bar{V}_W \tag{7}$$
$$= 74\ \mathrm{J}\cdot\mathrm{mol}^{-1}\cdot\mathrm{Å}^{-2}.$$

These two derivatives have different sign; thus, the rate of change with ASA is positive for the solute/water correction (see also Fig. 2), which implies that application of this correction will increase the slope of transfer Gibbs energy *versus* ASA, that is, the value calculated for the strength of the hydrophobic effect. On the other hand, the application of the organic solvent/solute correction can only *decrease* that value because the corresponding rate of change (Eq. (7) and Fig. 2) is *negative*. Note also that, owing to the lower molar volume of water ($\bar{V}_W \ll \bar{V}_S$), the absolute value of the rate of change with ASA is much larger for the solute/water correction than for the organic solvent/solute correction (Eqs. (6) and (7) and Fig. 2). As a result, application of *both* corrections (as prescribed by Eq. (4)) will increase the calculated value of the strength of the hydrophobic effect. It must be emphasized, however, that this increase is to be *entirely* attributed to the solute/water correction.

Type C. We study the dissolution into water of a homologous series of liquid hydrocarbons (for instance, n-alkanes from n=1 to n=10) and we intend to calculate a value for the strength of the hydrophobic effect as the slope of a plot of dissolution Gibbs energy *versus* hydrocarbon ASA. This is the type of data considered by Sharp et al. (1991b). We are dealing now with the transfer of substances from the pure liquid to the aqueous solution: that is, in every case the organic solvent and the solute are the same substance, $\bar{V}_2 = \bar{V}_S$, and the organic solvent/solute correction is strictly equal to zero. The only issue is then whether the solute/water correction is to be applied or not. As is obvious from Eq. (7) and Fig. 2 (and previously shown by Sharp et al.), application of the solute/water correction will significantly increase the value calculated for the strength of the hydrophobic effect.

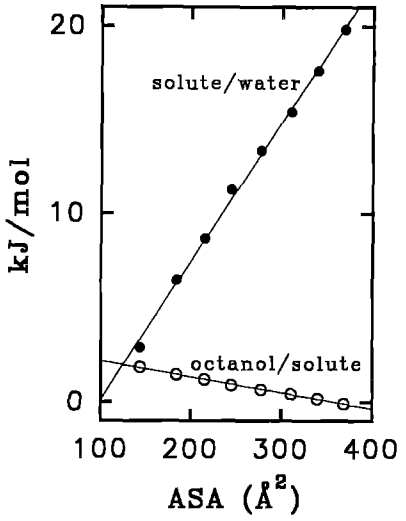

Fig. 2. Values of the molecular-size corrections for the transfer of n-alkanes (n = 1, 2, 3, 4, 5, 6, 7, 8) from n-octanol to water *versus* the accessible surface area of the alkane. Only the solute/water correction increases with the ASA value. In fact, application of the octanol/solute correction would decrease (slightly) a calculated value for the strength of the hydrophobic effect

The relation of the molecular-size corrections with Flory-Huggins theory

The molecular-size correction can be derived from the Flory-Huggins lattice theory (Flory 1941; Huggins 1941) and, within the context of this theory, its application would be required to extract the Flory binary interaction parameter from the experimental data (Chan and Dill 1994). According to the statistical-mechanical analysis recently reported by Chan and Dill (1994), the Flory-Huggins "size entropy" or "combinatorial entropy" (which would give rise to the molecular-size correction term) arises from the coupling of translational freedom to excluded volume: Flory-Huggins theory simply approximates this "coupling entropy" for solutions of polymeric solutes (or polymeric solvents). These authors analyze, in fact, a generalization of Flory-Huggins theory which includes empty lattice sites and, therefore, allows the pressure to be defined in the lattice model. They conclude that, for liquid solutions, the chemical potential given by the "traditional" Flory-Huggins theory (in which pressure is undefined) is a very good approximation to that derived from the generalized theory at any finite pressure. It is also shown (Chan and Dill 1994; Baker et al. 1993) that the results of Flory-Huggins theory are not artifacts caused by the mean-field approximations of the model (the Flory approximation for excluded volume and the Bragg-Williams approximation for the contact interactions), but correspond to the first term in the series expansion of the exact cluster lattice theory of Freed and colleagues (Freed 1985; Freed and Bawendi 1989).

Clearly, to the extent that solutions of apolar solutes in apolar solvents can be described on the basis of lattice models, the analysis of Flory-Huggins theory reported by Chan and Dill (1994) appears to support the application of the organic solvent/solute molecular-size correction. This is, in fact, consistent with the success of this correction in accounting for the chain-length-dependence of the n-alkane/water partition coefficient of benzene (De Young and Dill 1990; Fig. 1 in the present work).

However, the main issue of concern here is the value for the strength of the hydrophobic effect and, as has been shown above, this value is only significantly increased upon application of the solute/water molecular size correction. It is, therefore, very important to note that aqueous solutions of small apolar substances are very likely outside the domain of applicability of Flory-Huggins theory. In fact, the "traditional" Flory-Huggins theory was devised for concentrated solutions of apolar polymers in apolar solvents (although, it can be easily extended to polymer blends); the obvious shortcomings of its application to diluted aqueous solutions have been convincingly pointed out by several authors (Holtzer 1992; Abraham and Sakellaariou 1994) and there appears to be no need to review them here. We emphasize, nevertheless, that even the generalized treatment of Chan and Dill (1994) suggests that Flory-Huggins theory approximates the entropy of coupling of translational freedom to excluded volume only when polymeric solutes or solvents are involved (but not for other systems) and that size-dependent coupling entropies are not generally appropriate for rigid spherical or near spherical molecules, such as aminoacids, if polymeric solvents (alkanes, octanol, ...) are not involved.

To conclude, according to the present state of the theoretical research in the field, it appears that Flory-Huggins theory may provide the theoretical basis for the organic solvent/solute size correction but not for the application of the solute/water correction and the subsequent increase in the calculated value for the strength of the hydrophobic effect.

The analysis of dissolution processes in terms of hypothetical, ideal-gas, intermediate states (compact states)

Compact states (hypothetical ideal-gas states with the same density as the corresponding liquid state) have been employed as intermediates in several theoretical discussions on the relation between model transfer and protein folding thermodynamics (Privalov and Gill 1988; Makhatadze and Privalov 1990; Privalov and Makhatadze 1990, 1992). In 1991, Sharp, Nicholls, Friedman and Honig reported an analysis of dissolution processes in terms of this type of intermediate states (the SNFH analysis), which was intended to remove the translational entropy contributions from the transfer quantities. Given that translational entropy is related to the volume in which the molecules are allowed to move, we will (for lack of a better name) refer to the outcome of the SNFH analysis as "volume-entropy-corrected" transfer quantities.

As developed by Sharp et al. (1991b), the SNFH analysis leads to the molecular-size correction term in a manner which appears general and independent of specific models (such as the Flory-Huggins theory). Therefore, this type of derivation may be considered to provide a theoretical basis for the application of the solute/water size cor-

rection (and the concomitant increase in the value for the strength of the hydrophobic effect). However, the *status* of the SNFH analysis is controversial; thus, it has been strongly criticized on general grounds by some authors (Ben-Naim and Mazo 1993), while others (Lazaridis and Paulaities 1992, 1993; Tuñon et al. 1994; Lee 1994) have reported derivations of the molecular-size corrections which also seem to be based on translational entropy arguments. A critical assessment and generalization the analysis of dissolution processes in terms of compact intermediate states is given below, while other derivations of the molecular-size ratio term will be discussed in a subsequent section of this work.

The general scheme (Fig. 3) *for the analysis of gas and liquid dissolution.* We will analyze the dissolution of a liquid or gaseous solute (component 2) in a liquid solvent (component 1) at a given temperature and pressure. Following Sharp et al. (1991b), we will consider two different pathways to carry out this process (Fig. 3):

Pathway A. The actual dissolution process in which the *pure solute* (P2) is mixed with the pure *liquid solvent* (L1) to give the liquid mixture (LM): P2 + L1 → LM.

Pathway B. A hypothetical pathway that involves several steps:

B1) intermolecular interactions in the pure solute and solvent are switched off to form ideal-gas states with the same volume as the corresponding pure solute or solvent phases ("compact" states: C2 and C1): P2 → C2 and L1 → C1. Note that the pure solute (P2) may be either a liquid or a gas; in the latter case, it is taken to be a real gas in which intermolecular interactions are present [the ideal-gas approximation for the pure gaseous solute (which would imply that P2 ≡ C2) will only be made at a later stage in the analysis]. Note also that compact states are hypothetical states; that is, they are mental constructions (consider, for instance, the compact states corresponding to the liquid solvent or to a liquid solute: we are assuming the absence of intermolecular interactions and, hence, ideal-gas behavior, at liquid densities).
B2) mixing of the compact states to form a compact, ideal-gas mixture (CM) with the same volume as the liquid mixture (note that all volume changes occur in this step): C2 + C1 → CM.
B3) intermolecular interactions are connected in the compact mixture to yield the liquid mixture: CM → LM.

We will employ the following nomenclature. The Gibbs energy changes will be specified by indicating the corresponding step within brackets. Volumes (V), molar or partial molar volumes ($\bar{V}$), pressures (p), chemical potentials (μ), number densities (number of particles per unit volume, ρ) and molar fractions (X), will be labeled with a superscript and a subscript; the superscript specifies the state (P2, L1, LM, C2, C1 or CM) and the subscript indicates the component (1 or 2). The subscript will be omitted when unnecessary; for instance, the chemical potential of the solute in the compact mixture is μ_2^{CM}, while the molar volume of the solvent in the pure solvent phase is simply $\bar{V}^{L1}$.

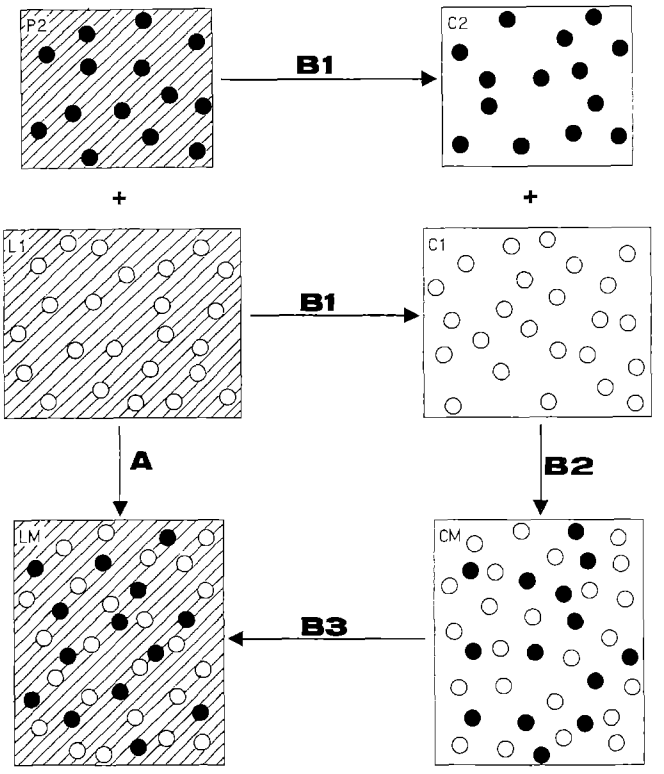

Fig. 3. Analysis of a solute dissolution process in terms of hypothetical, ideal-gas intermediate states (compact states). The absence of shading indicates that intermolecular interactions have been (mentally) switched off. Key to the symbols (see text for additional details): P2, pure solute; L1, pure liquid solvent; LM, liquid mixture; C2, solute compact state; C1, solvent compact state; CM, compact mixture; (●), a solute molecule; (○), a solvent molecule; (A), the actual dissolution process (pathway A); (B1, B2, B3), the several steps of a hypothetical way to carry out the dissolution (pathway B, see text for details)

Without any loss of generality we will assume that the actual mixing process (P2 + L1 → LM) takes place at the standard pressure: p° = 1 atm. However, the pressures of the compact states are given by the ideal-gas equation of state and, therefore, are not equal to the standard pressure. For future reference, the expressions for the volumes, molar volumes, number densities and pressures of the states involved in the scheme of Fig. 3 are given in Table 1. In this table, as well as in the following equations, n_1 and n_2 stand for the number of moles of solvent and solute, respectively, and N_0 is the Avogadro's number. Two types of equal to symbol have been used in the equations in Table 1. The symbol $\ll = \gg$ indicates a true thermodynamic equality (sometimes, under the ideal-gas approximation), while the symbol $\ll \overset{*}{=} \gg$ is used to connect two expressions that are equal only by virtue of the definition of compact states.

The derivation of the expression for volume-entropy-corrected transfer Gibbs energy of the solute. We first equate the total Gibbs energy changes along pathways A and B in Fig. 3:

$$\Delta G\{P2 + L1 \rightarrow LM\} = \Delta G^V + \Delta G\{C2 + C1 \rightarrow CM\} \quad (8)$$

Table 1. Expressions for the volumes, molar volumes, partial molar volumes, number densities and pressures of the states of Fig. 3

State	Volumes	Number densities	Pressures
Pure solute, P2	$V^{P2} = n_2 \cdot \bar{V}^{P2}$	$\rho^{P2} = N_0/\bar{V}^{P2}$	$p^{P2} \equiv p^\circ$
Compact solute state, C2	$V^{C2} = n_2 \cdot \bar{V}^{C2} \stackrel{*}{=} V^{P2}$ $\bar{V}^{C2} = RT/p^{C2}$ $\bar{V}^{C2} \stackrel{*}{=} \bar{V}^{P2}$	$\rho^{C2} = N_0/\bar{V}^{C2} \stackrel{*}{=} \rho^{P2}$	$P^{C2} = RT/\bar{V}^{C2}$ $p^{C2} \stackrel{*}{=} RT/\bar{V}^{P2}$ $p^{C2} \neq p^\circ$
Liquid solvent, L1	$V^{L1} = n_1 \cdot \bar{V}^{L1}$	$\rho^{L1} = N_0/\bar{V}^{L1}$	$p^{L1} \equiv p^\circ$
Compact solvent state, C1	$V^{C1} = n_2 \cdot \bar{V}^{C1} \stackrel{*}{=} V^{L1}$ $\bar{V}^{C1} = RT/p^{C1}$ $\bar{V}^{C1} \stackrel{*}{=} \bar{V}^{L1}$	$\rho^{C1} = N_0/\bar{V}^{C1} \stackrel{*}{=} \rho^{L1}$	$p^{C1} = RT/\bar{V}^{C1}$ $p^{C1} \stackrel{*}{=} RT/\bar{V}^{L1}$ $p^{C1} \neq p^\circ$
Liquid mixture, LM	$V^{LM} = n_1 \cdot \bar{V}_1^{LM} + n_2 \cdot \bar{V}_2^{LM}$	$\rho_1^{LM} = N_0 \cdot n_1/V^{LM}$ $\rho_2^{LM} = N_0 \cdot n_2/V^{LM}$	$p^{LM} \equiv p^\circ$
Compact mixture, CM	$V^{CM} \stackrel{*}{=} V^{LM}$ $V^{CM} \stackrel{*}{=} n_1 \cdot \bar{V}_1^{LM} + n_2 \cdot \bar{V}_2^{LM}$ $V^{CM} = n_1 \cdot \bar{V}_1^{CM} + n_2 \cdot \bar{V}_2^{CM}$ $\bar{V}_1^{CM} = \bar{V}_2^{CM} = RT/p^{CM}$ $\bar{V}_1^{CM} \neq \bar{V}_1^{LM}\ \ \bar{V}_2^{CM} \neq \bar{V}_2^{LM}$	$\rho_1^{CM} = N_0 \cdot n_1/V^{CM} \stackrel{*}{=} \rho_1^{LM}$ $\rho_2^{CM} = N_0 \cdot n_2/V^{CM} \stackrel{*}{=} \rho_2^{LM}$	$p^{CM} = (n_1 + n_2) \cdot RT/V^{CM}$ $p^{CM} \stackrel{*}{=} (n_1 + n_2) \cdot RT/V^{LM}$ $p^{CM} \neq p^\circ$

where ΔG^V is given by:

$$\Delta G^V = \Delta G\{P2 \rightarrow C2\} + \Delta G\{L1 \rightarrow C1\} + \Delta G\{CM \rightarrow LM\}. \quad (9)$$

The enthalpy change upon ideal-gas mixing at constant temperature is zero and, therefore,

$$\Delta G\{C2 + C1 \rightarrow CM\} = -T \cdot \Delta S\{C2 + C1 \rightarrow CM\} \quad (10)$$

where $\Delta S\{C2 + C1 \rightarrow CM\}$ is the corresponding, ideal-gas mixing entropy, which may be expressed in terms of the volume changes (Sharp et al. 1991b; Ben-Naim 1987):

$$\Delta S\{C2 + C1 \rightarrow CM\} = R \cdot (n_1 \cdot \ln(V^{CM}/V^{C1}) + n_2 \cdot \ln(V^{CM}/V^{C2})). \quad (11)$$

The SNFH analysis implies that the ΔG^V term describes all changes in interaction Gibbs energy upon dissolution and the unitary Gibbs energy change per mole of solute ($\Delta\bar{G}_2^V$) corresponding to this interaction term (ΔG^V),

$$\Delta\bar{G}_2^V = \left(\frac{\partial \Delta G^V}{\partial n_2}\right)_{T,p,n_1} \quad (12)$$

is devoid of translational entropy contributions. Taking Eq. (8) into account, $\Delta\bar{G}_2^V$ is given by,

$$\Delta\bar{G}_2^V = \Delta\bar{G}_2\{P2 \rightarrow LM\} - \Delta\bar{G}_2\{C2 \rightarrow CM\} \quad (13)$$

where,

$$\Delta\bar{G}_2\{P2 \rightarrow LM\} = \left(\frac{\partial \Delta G\{P2 + L1 \rightarrow LM\}}{\partial n_2}\right)_{T,p,n_1} = N_0 \cdot \left(\mu_2^{LM} - \mu^{P2}\right) \quad (14)$$

$$\Delta\bar{G}_2\{C2 \rightarrow CM\} = \left(\frac{\partial \Delta G\{C2 + C1 \rightarrow CM\}}{\partial n_2}\right)_{T,p,n_1} = N_0 \cdot \left(\mu_2^{CM} - \mu^{C2}\right). \quad (15)$$

The partial derivatives at constant pressure shown in Eqs. (12) and (15) require some comments, because they involve Gibbs energy changes corresponding to processes which do not occur at constant pressure (see the pressures of the states in Table 1). In fact, these derivatives are taken at constant *pressures of states;* that is, we differentiate with respect to the number of moles of solute involved in the process (n_2), keeping constant (besides the number of moles of solvent and the temperature) *the pressures of all the states involved in the process in the values given in Table 1.* For a given state, this type of differentiation yields zero if no solute is present (L1 and C1); otherwise (P2, C2, LM and CM), it yields the chemical potential of the solute (times the Avogadro's number). Hence, $\Delta\bar{G}_2\{P2 \rightarrow LM\}$ and $\Delta\bar{G}_2\{C2 \rightarrow CM\}$ represent the Gibbs energy associated with the transfer of a mole of solute between the specified states. Note that this "constant pressures of states" condition also applies to the derivative in Eq. (18) below and to the derivatives leading to Eqs. (B.9) through (B.11) in appendix B. Note also that we are using chemical potentials defined per molecule (as is usual in statistical mechanical treatments), rather than per mole (as is usual in phenomenological thermodynamics treatments).

For a saturated solution of solute, the transfer equilibrium condition ($\mu^{P2} \stackrel{e}{=} \mu_2^{LM}$) holds, therefore $\Delta\bar{G}_2\{P2 \rightarrow LM\} \stackrel{e}{=} 0$ and,

$$\Delta\bar{G}_2^V \stackrel{e}{=} -\Delta\bar{G}_2\{C2 \rightarrow CM\} \quad (16)$$

Taking Eq. (10) and the first equality in Eq. (15) into account, Eq. (16) may be written as:

$$\Delta\bar{G}_2^V \stackrel{e}{=} T \cdot \Delta\bar{S}_2\{C2 \rightarrow CM\} \tag{17}$$

where $\Delta\bar{S}_2\{C2 \rightarrow CM\}$ is the change in partial molar entropy upon transferring the solute from the compact state (C2) to the compact mixture (CM) and can be calculated by differentiation from Eq. (11):

$$\Delta\bar{S}_2\{C2 \rightarrow CM\} = \left(\frac{\partial\, \Delta S\{CS + C1 \rightarrow CM\}}{\partial\, n_2}\right)_{T,p,n_1} \tag{18}$$

$$= R \cdot (\ln(V^{CM}/V^{C2}) + (n_1 + n_2) \cdot (\bar{V}_2^{CM}/V^{CM}) - 1).$$

Now, noting that $\bar{V}_2^{CM} = RT/p^{CM}$ and that $p^{CM} = (n_1+n_2)RT/V^{CM}$ (see Table 1), we find that $(n_1+n_2) \cdot (\bar{V}_2^{CM}/V^{CM}) = 1$. Therefore,

$$\Delta\bar{S}_2\{C2 \rightarrow CM\} \tag{19}$$

$$= R \cdot \ln(V^{CM}/V^{C2}) \stackrel{*}{=} -R \cdot \ln(\rho_2^{LM}/\rho^{P2})$$

where we have used the compact state definitions ($V^{CM} \stackrel{*}{=} V^{LM}$ and $V^{C2} \stackrel{*}{=} V^{P2}$) and the relationships between volume and number densities (Table 1). Substitution into Eq. (17) yields,

$$\Delta\bar{G}_2^V \stackrel{e}{=} \stackrel{*}{=} -RT \cdot \ln(\rho_2^{LM}/\rho^{P2}) \tag{20}$$

which gives the volume-entropy-corrected, transfer Gibbs energy in terms of measurable quantities. Note that Eq. (20) is valid by virtue of the compact states definitions ($\stackrel{*}{=}$) and only holds for the number densities at equilibrium ($\stackrel{e}{=}$).

The treatment given above is more general than that previously employed by Sharp et al. (1991b). Thus, we have not specified whether the pure solute is a liquid or a real gas, we have not assumed volume additivity in the actural mixing process (pathway A) and we have not taken the high-dilution limit for the liquid mixture. Otherwise, we have closely followed the same steps as these authors did (although our account is somewhat more detailed) and we find that the molecular-size correction term $(-RT \cdot (1-r))$ does **not** appear in the final expression for the volume-entropy-corrected transfer Gibbs energy.

The soundness of this result may be checked in several ways; for instance, Eqs. (15) and (16) lead to:

$$\Delta\bar{G}_2^V \stackrel{e}{=} -N_0 \cdot (\mu_2^{CM} - \mu^{C2}) \tag{21}$$

For an ideal gas (C2) and for a perfect gas mixture (CM), the chemical potentials are given by (Denbigh 1981):

$$\mu^{C2} = \mu_2^\circ + kT \cdot \ln p^{C2} = \mu_2^\circ + kT \cdot \ln(kT \cdot \rho^{C2}) \tag{22}$$

$$\mu_2^{CM} = \mu_2^\circ + kT \cdot \ln(X_2^{CM} \cdot p^{CM}) \tag{23}$$

$$= \mu_2^\circ + kT \cdot \ln(kT \cdot \rho_2^{CM})$$

where we have used some of the equations in Table 1, and μ_2° is the chemical potential of the pure, ideal-gas solute at the standard pressure. Substitution of Eqs. (22) and (23) into Eq. (21) (and use of $\rho^{C2} \stackrel{*}{=} \rho^{P2}$ and $\rho_2^{CM} \stackrel{*}{=} \rho_2^{LM}$) yields Eq. (20).

Also, Eq. (20) can be arrived at from Eq. (21) by using the statistical-mechanical expressions for the chemical potentials (see, for instance, Ben-Naim 1993):

$$\mu^{C2} = -kT \cdot \ln q_2 + kT \cdot \ln(\rho^{C2} \cdot \Lambda_2^3) \tag{24}$$

$$\mu_2^{CM} = -kT \cdot \ln q_2 + kT \cdot \ln(\rho_2^{CM} \cdot \Lambda_2^3) \tag{25}$$

where q_2 is the internal partition function of a solute molecule (including the rotational, vibrational, electronic and nuclear degrees of freedom) and Λ_2 stands for its de Broglie's thermal wavelength.

Thus, there appears to be little doubt about the validity of Eq. (20), which suggests that the previous analysis which led to a molecular-size correction contains an error. The error (a rather subtle one, in fact) is exposed and briefly discussed in Appendix A. It must also be emphasized that the volume-entropy-corrected transfer Gibbs energy given by Eq. (20) is numerically equal to the local transfer Gibbs energy derived from Ben-Naim's approach (see Appendix B). This equality, however, does not hold for other transfer thermodynamic quantities, although the differences appear to be minor (Appendix B).

The relation between the molecular-size-ratio term and the "ideal-gas-like", entropic contribution from the solvent

As shown in Appendix A, the derivation of the molecular-size correction term in the SNFH analysis is due to the use of the liquid molar volume in an ideal-gas entropy equation (Eq. (18)). This use is obviously incorrect given that, once intermolecular interactions have been switched off, we obtain compact states with all the ideal-gas properties (including ideal-gas partial molar volumes). On the other hand, our analysis suggests that we should be able to "derive" the molecular-size-ratio term if we use statistical-mechanical arguments to separate an "ideal-gas-like" (translational) entropic contribution and, at the same time, we avoid the use of intermediate ideal-gas states (so that we are not forced to insert ideal-gas partial molar volumes in the ideal-gas-like equations!). In fact, this procedure appears to be behind several recent derivations of the molecular-size-ratio term (Lazaridis and Paulaities 1992, 1993; Tuñon et al. 1994; Lee 1994) and is illustrated below:

The chemical potential of the solvent (component 1) and solute (component 2) in a solution may be written as (Ben-Naim 1978, 1992):

$$\mu_1 = \mu_1^* + kT \cdot \ln(\rho_1 \cdot \Lambda_1^3) \tag{26}$$

$$\mu_2 = \mu_2^* + kT \cdot \ln(\rho_2 \cdot \Lambda_2^3) \tag{27}$$

where ρ_i and Λ_i are the number density and the thermal wavelength of the component i, and μ_i^*stands for its pseudochemical potential, that is, the work associated with the insertion of a molecule of i to a fixed center-of-mass position in the system. The liberation term, $kT \cdot \ln(\rho_i \cdot \Lambda_i^3)$, is the work associated with the removal of the fixed-position constraint and, obviously, takes into account the translational freedom of the i molecule. Note that the liberation term is mainly entropic and exactly the same as the corresponding term for an ideal gas phase (compare Eqs. (26) and (27) with Eqs. (24) and (25)). [Note also that, since

we are no longer analyzing the scheme of Fig. 3, we are now using a simpler and more conventional notation].

Application of the Euler theorem to the Gibbs energy of the system gives $G = N_1\mu_1 + N_2\mu_2$, and using Eqs. (26) and (27):

$$G = \{N_1\mu_1^* + N_2\mu_2^*\} + G^{lib} \tag{28}$$

where G^{lib} might be considered as the liberation Gibbs energy of the entire system

$$G^{lib} = N_1\,kT \cdot \ln(\rho_1 \cdot \Lambda_1^3) + N_2\,kT \cdot \ln(\rho_2 \cdot \Lambda_2^3) \tag{29}$$

and represents our "ideal-gas-like" translational contribution. (N_1 and N_2 stand for the number of solvent and solute molecules: $N_1 = N_0 \cdot n_1$, $N_2 = N_0 \cdot n_2$).

The chemical potential is the molecular partial Gibbs energy; hence, using Eqs. (28) and (29),

$$\begin{aligned}\mu_2 &= \left(\frac{\partial\{N_1\mu_1^* + N_2\mu_2^*\}}{\partial N_2}\right)_{T,p,N_1} + \left(\frac{\partial G^{lib}}{\partial N_2}\right)_{T,p,N_1} \\ &= \left(\frac{\partial\{N_1\mu_1^* + N_2\mu_2^*\}}{\partial N_2}\right)_{T,p,N_1} \\ &\quad + kT\cdot\left(1 - \frac{v_2}{V}(N_1 + N_2)\right) + kT\cdot\ln(\rho_2\cdot\Lambda_2^3)\end{aligned} \tag{30}$$

where V is the volume of the system and v_2 is the molecular partial volume of the solute (in the solution, not in a hypothetical ideal-gas phase). For a diluted solution $V \cong N_1 v_1$ and $N_1 + N_2 \cong N_1$ and the chemical potential of the solute reduces to:

$$\begin{aligned}\mu_2 &= \left(\frac{\partial\{N_1\mu_1^* + N_2\mu_2^*\}}{\partial N_2}\right)_{T,p,N_1} \\ &\quad + kT\cdot(1-r) + kT\cdot\ln(\rho_2\cdot\Lambda_2^3)\end{aligned} \tag{31}$$

where r is the molecular-size ratio ($r = v_2/v_1$). Of course, Eq. (31) simply provides one of the several possible dissections of the pseudochemical potential; thus, comparison of Eqs. (31) and (27) yields:

$$\mu_2^* = \left(\frac{\partial\{N_1\mu_1^* + N_2\mu_2^*\}}{\partial N_2}\right)_{T,p,N_1} + kT\cdot(1-r). \tag{32}$$

The above analysis (Eqs. (28–32)) follows approximately the interesting treatment recently reported by Tuñon et al. (1994). The important point to note is, however, that the molecular-size ratio term will be "exposed" as part of the chemical potential of the solute (or as part of its partial molar entropy) if we start with any valid statistical-mechanical expression that considers separately an ideal-gas-like, translational contribution to the thermodynamic quantities. In fact, this separation can be achieved in many ways; for instance: a) from Ben-Naim's splitting of the chemical potential into a pseudochemical potential *plus* a (ideal-gas-like) liberation term (Tuñon et al. 1994 and derivation given above); b) from the general expression for the canonical partition function of the system (Lee 1994); c) from the correlation expansion for the entropy of the system (Lazaridis and Paulaities 1993).

It should be clear that, regardless of the specific method used to separate the ideal-gas-like contribution, the molecular-size ratio (–kTr) which will appear in the final expression for the chemical of the solute at high dilution (as in Eqs. (31) and (32)) simply reflects the effects of solute addition at constant temperature and pressure on the ideal-gas-like, entropic contribution from the solvent to the Gibbs energy of the system; this effect is obviously mediated by the change in volume that accompanies solute addition at constant T and *pressure*. This is most clearly seen in the context of Ben-Naim's splitting of the chemical potential (Eqs. (26) and (27)). Thus, consider the liberation Gibbs energy of N_1 molecules of solvent at a given temperature and *pressure* in the absence (pure solvent) and presence of a molecule of solute:

$$\{N_1\mu_1\}^{lib} = N_1 kT\cdot\ln\left(\frac{N_1\cdot\Lambda_1^3}{V}\right) \tag{33}$$

$$\{N_1\mu_1\}^{lib}_{N_2=1} = N_1 kT\cdot\ln\left(\frac{N_1\cdot\Lambda_1^3}{V + v_2}\right) \tag{34}$$

where we have taken into account that, upon addition of a solute molecule at constant T and p, the volume of the system increases by an amount equal to the solute molecular volume, v_2 (see appendix C). The effect of solute addition on the solvent liberation Gibbs energy (the solvent, ideal entropic contribution) is then:

$$\begin{aligned}&\{N_1\mu_1\}^{lib}_{N_2=1} - \{N_1\mu_1\}^{lib} \\ &= -N_1\,kT\cdot\ln(1 + v_2/V) \cong -kTr\end{aligned} \tag{35}$$

where we have used (since $v_2/V \ll 1$) $\ln(1 + v_2/V) \cong v_2/V$ and $V \cong N_1 v_1$.

At this point, it must be emphasized that the effect of solute addition on the solvent liberation Gibbs energy is a legitimate part of the solute chemical potential, given that this may be defined as the change in the Gibbs energy *of the entire system* that accompanies the addition of a solute molecule at constant T and p (see appendix C). It is also a legitimate part of the solute pseudo-chemical potential, which is the change in the Gibbs energy of *the entire system*, but excluding the solute liberation Gibbs energy: $kT\cdot\ln(\rho_2\Lambda_2^3)$. From this point of view, the solute chemical and pseudo-chemical potentials (as well as Ben-Naim's solvation quantities, which are defined in terms of pseudo-chemical potentials) *need no correction.*

On the other hand, it might be adduced that the real issue is, in fact, whether a correction is required prior to the use of model transfer data for the specific purpose of estimating the hydrophobic contribution to protein folding (or other biologically relevant processes). In particular, it would perhaps seem that some correction for the effects associated with the volume change of the model transfer process is advisable, since this volume change is expected to be larger (on a comparable basis) than that associated with folding/unfolding processes (see the "lattice cartoon" in Fig. 4). Furthermore, it could be argued that this correction is given by the molecular-size ratio term (which would cause a significant increase in the calculated value for the strength of the hydrophobic effect). As explained

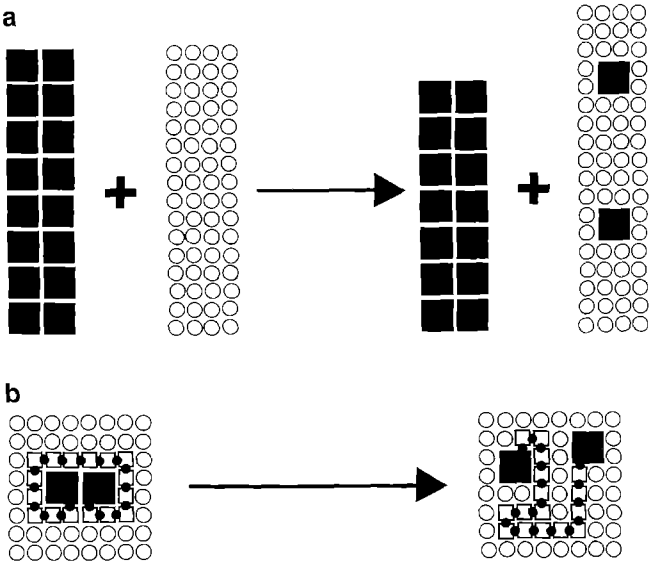

Fig. 4a, b. Two-dimensional, lattice cartoon illustrating volume changes in model transfer and protein unfolding processes. **a**, transfer of two apolar solute molecules (*large black squares*) from the pure solute to aqueous solution (○ ≡ solvent molecule); note the volume changes. **b**, unfolding of a very simplistic protein model; the "protein" consists of two apolar residues (*large black squares*) joined by a chain of hydrophilic residues (*small open squares*). Unfolding exposes the apolar residues to the solvent, but does not involve volume changes

below, however, there is (at least) one argument that highly disfavors this point of view (somewhat similar arguments to that given below, but based on a mean-field continuum representation of the solvent, have been recently advanced by Rashin and Bukatin (1994a, 1994b)).

The molecular-size-ratio term (Eq. (35)) gives the change in the solvent, "ideal-gas-like" entropic contribution to the Gibbs energy of the system caused by the volume change associated with solute addition at constant T and pressure. In most cases, this volume change will be a volume increase, mainly due to the *repulsive solute-solvent interactions*: to a good degree of approximation, the volume of the system increases at constant T and pressure because the solvent molecules are not allowed to enter a "hard-core region" of the approximate size of the solute molecule. That is, to a good degree of approximation, the *actual volume accessible to the solvent molecules* does not change upon solute addition at constant T and pressure. From this point of view, *the overall effect of solute addition on solvent, ideal entropic contribution is, in fact, close to zero.* The reason is that the change in solvent liberation Gibbs energy associated with the increase in the *volume of the system* from V to $V + v_2$ (–kTr in Eq. (35)) is nearly canceled out by the effect of the repulsive solute-solvent interaction which will decrease the actual *volume accessible* to the solvent molecules from $V + v_2$ to approximately V and, hence, will change the solvent liberation Gibbs energy by about kTr. Clearly, this kTr contribution to the chemical potential is included in equations (31) and (32), but as part of the $\partial\{N_1\mu_1^* + N_2\mu_2^*\}/\partial N_2$ term (that is, it is not "exposed" as the –kTr term).

Consider now a protein unfolding process and the volume change due to the concomitant exposure to the solvent of a given group previously buried in the interior of the native structure. For compact protein structures (see Fig. 4), the group contributes to the protein-solvent repulsive interactions, both in the folded and unfolded states (note that the group contributes to the "hard-core" volume of a compact protein, even when it is buried in the native structure; see Fig. 4). As a result, the volume change associated with the exposure of the group and the concomitant change in solvent, ideal entropic contribution are expected to be rather small (see Fig. 4). Therefore, we conclude that in both, the model transfer process and the protein unfolding process, the change in the solvent, ideal entropic contribution associated with the exposure of a molecule (or group) is close to zero; hence, *no correction for this contribution is required.* It is important to note that the above argument would still work if we considered the whole entropic contribution that arises from the volume accessible to the solvent molecules (and not just an "ideal part").

The effect of the volume-corrections attributed to solute-solvent, repulsive interactions

The above considerations clearly argue against the need to apply corrections for the solvent, entropic contributions associated with changes in the volume of the system; on the other hand, they emphasize the different role played by intermolecular *repulsive* interactions in model transfer and protein folding/unfolding processes. Given that repulsive interactions may be described (to a good degree of approximation) in terms of hard-core sizes, it might still be adduced that some correction related to the size (volume) of the solute should be applied to the model transfer data (even if that correction is not given by the solute/water molecular-size ratio term). In connection with this, it is of interest that general considerations (Ben-Naim and Mazo 1993), as well as the analysis of some simple models (Ben-Naim and Mazo 1993, Ben-Naim 1994, Perkyns and Pettit 1994a, b) support the view that Ben-Naim's solvation quantities may be expressed as a sum of a term that depends on solute area plus a term that depends on solute volume:

$$\Delta\bar{G}_2^* = a \cdot V_2 + b \cdot A_2 \qquad (36)$$

where V_2 is the solute volume and A_2 is its area. A detailed discussion on expressions such as Eq. (36) is outside the scope of this work [which deals with molecular-size corrections of the $RT \cdot (1 - r)$ type]. However, we wish to emphasize two important points which, surprisingly, appear to have been missed in some recent discussions: 1) the important issue is **not** whether a given contribution is proportional to solute volume or to solute area, but whether the contribution arises from intermolecular solute-solvent repulsive interactions; 2) in any case, and according to several analyses (Ben-Naim and Mazo 1993; Ben-Naim 1994; Perkins and Pettit 1994a, b), the coefficient of volume in Eq. (36) appears to be a positive number; thus, contrary to the solute/water molecular-size correction, *correction for*

the volume dependence in Eq. (36) will only decrease the value calculated for the strength of the hydrophobic effect. This second point can be reasoned as follows:

Since, for an homologous series of substances, volume and area usually show an acceptably linear correlation (see for instance Eq. (5) in the present work), an "uncorrected" value for the strength of the hydrophobic effect calculated as the slope of a plot of $\Delta\bar{G}_2^*$ in water versus solute area would be given by,

$$\text{"uncorrected"} \quad \partial \Delta\bar{G}_2^*/\partial A_2 = \beta a + b \tag{37}$$

where β is the slope of a volume versus area plot: $\beta = \partial V_2/\partial A_2$.

On the other hand, we might adopt the view that the volume term in Eq. (36) arises from the solute-solvent repulsive interactions in the model transfer processes and that this type of contribution would not occur in protein unfolding (since repulsive solute-solvent interaction may be expected to be present in both the folded and the unfolded state: Fig. 4). Then, although the volume term is a legitimate part of the solvation Gibbs energy, we might consider that its "removal" is advisable for the specific purpose of estimating the hydrophobic contribution to protein folding thermodynamics. Accordingly a "corrected" value for the strength of the hydrophobic effect would be given by,

$$\text{"corrected"} \quad \partial[\Delta\bar{G}_2^* - aV_2]/\partial A_2 = b \tag{38}$$

We will not discuss here the validity of the view that leads to the corrected value of Eq. (38), but simply point out that, since a is positive (and so is β in Eq. (37)) we have that $b < a\beta + b$ and the volume-correction would only decrease the value calculated for the strength of the hydrophobic effect (that is, application of the correction would lead to *lower* estimates for the hydrophobic contribution to protein folding thermodynamics).

Concluding remarks

A large increase in the calculated value for the strength of the hydrophobic contribution would arise as a result of the application of the solute/water molecular-size correction. Molecuar-size corrections have been derived on the basis of: 1) the Flory-Huggins lattice theory; 2) ideal translational entropy arguments which stem from the analysis of dissolution processes in terms of ideal-gas, intermediate states, as originally developed by Sharp et al. (1991b). The recent work of Chan and Dill (1994) indicates that the Flory-Huggins theory may provide the theoretical basis for the organic solvent/solute size correction but not for the application of the solute/water correction and the subsequent increase in the calculated value for the strength of the hydrophobic effect. In addition, we have shown in this work that, contrary to the original claims, the analysis of dissolution in terms of ideal-gas intermediate states does *not* lead to the molecular-size correction terms but only to expressions similar to those obtained from Ben-Naim's statistical-mechanical approach.

Several points of view are possible regarding the estimation of the hydrophobic contribution to protein folding (and other biologically relevant processes) from model transfer data. Thus, we might use the often-quoted value of about 100 J/mol per $Å^2$ of apolar ASA exposed to the aqueous medium, which can be derived from the analysis of liquid hydrocarbon dissolution. On the other hand, it may be argued that a crystalline solid is a better model for a protein interior than a liquid hydrocarbon (Murphy and Gill 1991); the use of solid hydrocarbons as the initial state of the dissolution process would lead (Makhatadze and Privalov 1995) to a significantly higher value for the strength of the hydrohobic effect: about 180 $J \cdot mol^{-1} \cdot Å^{-2}$. Also, it may be deemed more convenient to consider separately the interactions in the aqueous and the non-aqueous media; the apolar group-water interactions (hydration) might then be modeled on the basis of gas dissolution thermodynamics (Privalov and Makhatadze 1993; Makhatadze and Privalov 1993; Privalov and Makhatadze 1995), which would lead to a value close to zero $J \cdot mol^{-1} \cdot Å^{-2}$ for this hydration contribution [of course, this hydration contribution would not include the van der Waals interactions of the apolar groups in the protein interior]. Alternatively, Ben-Naim (1990) has proposed that the relation between protein folding and transfer processes with small model compounds should be based on the concept of conditional solvation Gibbs energies. Regardless of the specific point of view adopted, however, the analyses offered or discussed in this work disfavor the application of the solute/water molecular-size correction and the consequent revision upwards of the above estimates of the strength of the hydrophobic effect.

Note added in proof

The analyses offered in this work disfavor the application of a solute/water, molecular size correction. They, however, do not rule out that other types of corrections could be required prior to the use of model transfer data for the purpose of estimating hydrophobic contributions to protein folding. The theoretical work of Chan and Dill (1994) suggests the importance of the entropic contributions that arise from steric interferences between orientations of neighbor alkane molecules, an effect characteristic of a liquid alkane phase and, possibly, extraneous to protein folding. An attempt to estimate these entropic contributions has been recently carried out by this author on the basis of Pitzer's extension of the law of corresponding states [Sanchez-Ruiz JM (1995) An estimate of shape-related contributions to hydrophobic Gibbs energies. J Phys. Chem. 99:12076–12080]. The strength of the hydrophobic effect derived from liquid alkane dissolution was found to increase to about 170 $J \cdot mol^{-1} \cdot Å^{-2}$ upon correcting for the entropic contributions thus calculated, while a value of about 200 $J \cdot mol^{-1} \cdot Å^{-2}$ was obtained upon correcting for the whole solvation entropy of the liquid alkane. These values are, in fact, close to those suggested by studies of the effect of aliphatic mutations on protein stability.

Appendix A: The origin of the molecular-size correction in a previous theoretical analysis of dissolution processes in terms of intermediate, ideal-gas states (compact states)

The original SNFH analysis (Sharp et al. 1991b) apparently contains a (rather subtle) mistake, which will be discussed in some detail below. We will employ the terminology of the present work (Fig. 3 and Sect. 4).

The volume of the compact mixture is constrained to be equal to that of the liquid mixture ($V^{CM} \stackrel{*}{=} V^{LM}$) and, using the Euler theorem for the latter volume, we arrive at (Table 1):

$$V^{CM} \stackrel{*}{=} n_1 \cdot \bar{V}_1^{LM} + n_2 \cdot \bar{V}_2^{LM} \tag{A.1}$$

Direct application of the Euler theorem to the volume of the compact mixture yields (Table 1):

$$V^{CM} = n_1 \cdot \bar{V}_1^{CM} + n_2 \cdot \bar{V}_2^{CM} \tag{A.2}$$

Comparison between A1 and A2 lures us into believing that the partial molar volumes in the liquid mixture and the compact mixture are equal. For instance,

$$\bar{V}_2^{CM} = \bar{V}_2^{LM} \tag{A.3}$$

which is, apparently, the mistake in the original SNFH analysis. Note that intermolecular interactions are not present in the compact mixture which is, therefore, a perfect gas mixture as defined by Denbigh (1981); it can be shown (Denbigh 1981) that the partial molar volume of any substance in a perfect gas mixture equals RT divided by the pressure of the mixture. Thus, $\bar{V}_2^{CM}$ does not equal $\bar{V}_2^{LM}$, but is given by RT/p^{CM} as shown in Table 1 and employed in the derivation given in the text.

On the other hand, if we accepted as correct Eq. (A.3), then the $(n_1 + n_2) \cdot (\bar{V}_2^{CM}/V^{CM})$ term in Eq. (18) would not equal unity. In fact, assuming [as in the original SNFH analysis] that the solution is very diluted [that is, $n_1 + n_2 = n_1$ and $V^{CM} \stackrel{*}{=} n_1 \cdot \bar{V}_1^{LM}$] and that volume additivity holds for the mixing process [that is, $\bar{V}_1^{LM} = \bar{V}^{L1}$ and $\bar{V}_2^{LM} = \bar{V}^{L2}$], we would find that the above term equals the sought-for, molecular-size ratio:

$$(n_1 + n_2) \cdot (\bar{V}_2^{CM}/V^{CM}) = (\bar{V}^{L2}/\bar{V}^{L1}) = r \tag{A.4}$$

where we have recognized that the pure solute is a liquid by using L2 instead of P2. Hence, Eq. (18) in the text would become,

$$\Delta\bar{S}_2\{C2 \rightarrow CM\} = R \cdot (\ln(V^{CM}/V^{C2}) + r - 1) \stackrel{*}{=} -R \cdot \ln(\rho_2^{LM}/\rho^{L2}) - R \cdot (1 - r) \tag{A.5}$$

and substitution into Eq. (17) would yield,

$$\Delta\bar{G}_2^{V} \stackrel{e}{=} \stackrel{*}{=} -RT \cdot \ln(\rho_2^{LM}/\rho^{L2}) - RT \cdot (1 - r) \tag{A.6}$$

which is, in fact, equation (7) in Sharp et al. (1991b). In contrast to Eq. (20) in the text, Eq. (A.6) does contain the molecular-size correction term $(-RT \cdot (1 - r))$, but only as a result having used Eq. (A.3), which is not correct.

Possibly, the incorrectness of Eq. (A.3) requires further clarification. The partial molar volume of the solute is obtained by differentiating the total volume with respect to n_2 while keeping constant n_1, T and *pressure:*

$$\bar{V}_2^{CM} = \left(\frac{\partial V^{CM}}{\partial n_2}\right)_{n_1,T,p} \tag{A.7}$$

Equation (A.1) is not a thermodynamic identity (note the symbol $\stackrel{*}{=}$) and, in fact, it holds true owing to the fact that the compact mixture is constrained to have the same volume as the liquid mixture ($V^{CM} \stackrel{*}{=} V^{LM}$). Hence, $\bar{V}_2^{LM}$ may be expressed as a derivative of the volume of the compact mixture, but only if the constraint is maintained in the derivative; that is,

$$\bar{V}_2^{LM} = \left(\frac{\partial V^{CM}}{\partial n_2}\right)_{n_1,T,V^{CM} \stackrel{*}{=} V^{LM}} \tag{A.8}$$

For equation (A.3) to hold true, the partial derivatives in Eqs. (A.7) and (A.8) must be equal; they are not, however, since *the pressure does not remain constant when* n_2 *is varied while the volume of the compact mixture is forced to be equal to that of the liquid mixture.* The correct relationship between both derivatives can be easily derived from the known rules of partial differentiation:

$$\bar{V}_2^{CM} = \bar{V}_2^{LM} - \left(\frac{\partial V^{CM}}{\partial p}\right)_{T,n_1,n_2} \times \left(\frac{\partial p^{CM}}{\partial n_2}\right)_{n_1,T,V^{CM} \stackrel{*}{=} V^{LM}} \tag{A.9}$$

The compact mixture is a perfect gas mixture and obeys as a whole the ideal-gas equation of state (see Table 1), from whence the two derivatives of the second term in the right-hand-side of (A.9) may be easily evaluated:

$$\left(\frac{\partial V^{CM}}{\partial p}\right)_{T,n_1,n_2} = -V^{CM}/p^{CM} \tag{A.10}$$

$$\left(\frac{\partial p^{CM}}{\partial n_2}\right)_{n_1,T,V^{CM} \stackrel{*}{=} V^{LM}} = (RT - p^{CM} \cdot \bar{V}_2^{LM})/V^{CM}. \tag{A.11}$$

As indicated above, the derivative of pressure in A.9 is not zero (Eq. (A.11)). Substitution of A.10 and A.11 into A.9 recovers the correct value for the partial molar volume of the solute in the compact mixture: $\bar{V}_2^{CM} = RT/p^{CM}$.

Finally, it must be noted that the "pressure problem" does not arise with the pure compact states (C1 and C2) because the pressures, p^{C1} and p^{C2}, do not depend on n_2 (Table 1) and the pressure derivative analogous to that in Eq. (A.11) is zero. Therefore, we may write $\bar{V}^{C2} \stackrel{*}{=} \bar{V}^{P2}$ and $\bar{V}^{C1} \stackrel{*}{=} \bar{V}^{L1}$, as is shown in Table 1.

Appendix B: The relation between volume-entropy-corrected and Ben-Naim's local transfer quantities

Consider the scheme of Fig. 3. The chemical potential of the solute in the pure solute phase (P2) and liquid mixture (LM) may be written as:

$$\mu^{P2} = \mu^{*P2} + kT \cdot \ln(\rho^{P2} \cdot \Lambda_2^3) \tag{B.1}$$

$$\mu_2^{LM} = \mu_2^{*LM} + kT \cdot \ln(\rho_2^{LM} \cdot \Lambda_2^3) \tag{B.2}$$

where μ^{*P2} and μ_2^{LM} are the pseudochemical potentials of the solute in the pure phase and the liquid mixture; in general, pseudochemical potentials may be interpreted as the work associated with the addition of a molecule to a fixed center-of-mass position (R_o) in the system (Ben-Naim 1978, 1993) and are devoid of contributions arising from the molecule translational degrees of freedom and the indistinguishability from other particles. Ben-Naim's, *local* transfer quantities are defined as the changes in thermo-

dynamic quantities associated with the transfer of a molecule from a fixed position in a given environment to a fixed position in another environment. Thus, the local, transfer Gibbs energy for solute dissolution (expressed per mole of solute) is defined as:

$$\Delta\bar{G}_2\{P2 \rightarrow LM, \mathbf{R_0}\} = N_0 \cdot (\mu_2^{*LM} - \mu^{*P2}) \quad (B.3)$$

or, using Eqs. (B.1), (B.2) and (14):

$$\Delta\bar{G}_2\{P2 \rightarrow LM, \mathbf{R_0}\} = \Delta\bar{G}_2\{P2 \rightarrow LM\} - RT \cdot \ln(\rho_2^{LM}/\rho^{P2}). \quad (B.4)$$

The local, transfer Gibbs energy can be easily calculated from the equilibrium number densities, since, at equilibrium, $\Delta\bar{G}_2\{P2 \rightarrow LM\} \stackrel{e}{=} 0$, and, therefore,

$$\Delta\bar{G}_2\{P2 \rightarrow LM, \mathbf{R_0}\} \stackrel{e}{=} -RT \cdot \ln(\rho_2^{LM}/\rho^{P2}). \quad (B.5)$$

However, we do not need to assume equilibrium in order to obtain the general relation between the local and the volume-entropy-corrected transfer Gibbs energies, but simply eliminate $\Delta\bar{G}_2\{P2 \rightarrow LM\}$ between Eq. (B.4) and Eq. (13):

$$\Delta\bar{G}_2^V = \Delta\bar{G}_2\{P2 \rightarrow LM, \mathbf{R_0}\} - \Delta\bar{G}_2\{C2 \rightarrow CM\} + RT \cdot \ln(\rho_2^{LM}/\rho^{P2}) \quad (B.6)$$

and, using that $\Delta\bar{G}_2\{C2 \rightarrow CM\} = -T \cdot \Delta\bar{S}_2\{C2 \rightarrow CM\} = -RT \cdot \ln(V^{CM}/V^{C2})$ (see Eq. (19)),

$$\Delta\bar{G}_2^V = \Delta\bar{G}_2\{P2 \rightarrow LM, \mathbf{R_0}\} + RT \cdot \ln(V^{CM}/V^{C2}) + RT \cdot \ln(\rho_2^{LM}/\rho^{P2}) \quad (B.7)$$

Finally, using the compact state definitions ($V^{CM} \stackrel{*}{=} V^{LM}$ and $V^{C2} \stackrel{*}{=} V^{LM}$) and the relationships between volumes and number densities (Table 1), Eq. (B.7) reduces to:

$$\Delta\bar{G}_2^V \stackrel{*}{=} \Delta\bar{G}_2\{P2 \rightarrow LM, \mathbf{R_0}\} \quad (B.8)$$

which shows that the local and the volume-entropy-corrected transfer Gibbs energies are numerically equal *in general* (that is, not only in the equilibrium case).

Expressions for the volume-entropy-corrected, transfer enthalpy, entropy and heat capacity can now be obtained by temperature derivation: $\Delta\bar{H}_2^V = -T^2 \cdot (\partial[\Delta\bar{G}_2^V/T]/\partial T)$, $\Delta\bar{S}_2^V = -(\partial\Delta\bar{G}_2^V/\partial T)$ and $\Delta\bar{C}_{p,2}^V = -T \cdot (\partial^2\Delta\bar{G}_2^V/\partial T^2)$. It is important to note that this derivation procedure is not applied to Eq. (B.8) which is not a true thermodynamic equality (note the symbol $\ll \stackrel{*}{=} \gg$), but to Eq. (B.7). Note also that the above are partial derivatives at constant composition *and pressures of the states*; this implies that the ratio V^{CM}/V^{C2} in Eq. (B.7) [which, according to the relations of Table 1, is equal to $X_2^{LM} \cdot (p^{C2}/p^{CM})$] is to be taken as a constant in these derivations. The results are:

$$\Delta\bar{H}_2^V = \Delta\bar{H}_2\{P2 \rightarrow LM, \mathbf{R_0}\} - RT^2 \cdot \alpha^{P2} + RT^2 \cdot \alpha^{LM} \quad (B.9)$$

$$\Delta\bar{S}_2^V \stackrel{*}{=} \Delta\bar{S}_2\{P2 \rightarrow LM, \mathbf{R_0}\} - RT \cdot \alpha^{P2} + RT \cdot \alpha^{LM} \quad (B.10)$$

$$\Delta\bar{C}_{p,2}^V = \Delta\bar{C}_{p,2}\{P2 \rightarrow LM, \mathbf{R_0}\} + 2RT \cdot \alpha^{P2} + RT^2 \cdot \left(\frac{\partial\alpha^{P2}}{\partial T}\right)_p - 2RT \cdot \alpha^{LM} - RT^2 \cdot \left(\frac{\partial\alpha^{LM}}{\partial T}\right)_p \quad (B.11)$$

where α^{P2} and α^{LM} are the thermal expansion coefficients of the pure solute and the liquid mixture, although, for a diluted solution, the latter value may be approximated by that corresponding to the pure solvent: $\alpha^{LM} \cong \alpha^{L1}$.

The differences between the local and the volume-entropy-corrected transfer enthalpy, entropy and heat capacity are certainly minor, although perhaps not entirely negligible in many cases. For instance, the terms containing the expansion coefficient of the pure solute in Eqs. (B.9) through (B.11) would be equal to –RT, –R and 2R, if the pure solute is an ideal gas (since, in that case, $\alpha^{P2} = 1/T$). Also, the term containing the temperature-derivative of the expansion coefficient of the solvent (using the approximation $\alpha^{LM} \cong \alpha^{L1}$) in Eq. (B.11) is significant ($-7.4\ J \cdot K^{-1} \cdot mol^{-1}$ at 25 °C) when the solvent is water [water shows an atypical volume/temperature behavior and its expansion coefficient changes very rapidly with temperature, so that $(\delta\alpha^{L1}/\delta T)_p$ is very high: about $10^{-5}\ K^{-2}$ at 25 °C].

Appendix C: The chemical potential in the T,p,{N} ensemble

It is customary in some statistical-mechanical treatments to define the chemical potential in the T,V,{N} ensemble as the Hemholtz energy associated with the addition of a molecule to the system at constant temperature and volume. Thus, the chemical potential of the solute (component 2) is:

$$\mu_2[T,V,\{N\}] = A(T,V,N_1,N_2+1) - A(T,V,N_1,N_2) = -kT \cdot \ln\frac{Q(T,V,N_1,N_2+1)}{Q(T,V,N_1,N_2)} \quad (C.1)$$

where we have used the fundamental relation between Hemholtz energy and canonical partition function: $A = -kT \cdot \ln Q$.

In the T,p,{N} ensemble we define the chemical potential as the Gibbs energy change associated with the insertion of a molecule to the system at constant temperature and pressure:

$$\mu_2[T,p,\{N\}] = G(T,p,N_1,N_2+1) - G(T,p,N_1,N_2) = -kT \cdot \ln\frac{\Delta(T,p,N_1,N_2+1)}{\Delta(T,p,N_1,N_2)} \quad (C.2)$$

where we have used the fundamental relation between Gibbs energy and the isothermal-isobaric partition function: $G = -kT \cdot \ln\Delta$. It is often stated that (in the thermodynamic limit) the chemical potential defined in the T,V,{N} ensemble is identical to that defined in the T,p,{N} ensemble, provided that the volume of the systems in the first case (Eq. (C.1)) and the average volume of the ensemble in the second one (Eq. (C.2)) are equal. As shown below, however, this statement, does not emphasize the volume change associated with solute addition at constant T and p, and, therefore, may lead to confusion

when discussing volume-related contributions to thermodynamic quantities.

The isothermal-isobaric partition functions of Eq. (C.2) are given by,

$$\Delta(T,p,N_1,N_2+1)=\sum_V Q(T,V,N_1,N_2+1)\cdot e^{-pV/kT} \quad (C.3)$$

$$\Delta(T,p,N_1,N_2)=\sum_V Q(T,V,N_1,N_2)\cdot e^{-pV/kT} \quad (C.4)$$

Given that volume is a continuous variable, the sums in the above equations may be replaced by integrals times a constant with dimension of reciprocal volume (for details see Hill 1956), but we will not use this replacement here.

Volume is a fluctuating variable in the T, p, {N} ensemble; the ensemble average volumes for the systems with N_2+1 and N_2 molecules of solute are given by:

$$\langle V\rangle_{N_2+1}=\frac{\sum_V V\cdot Q(T,V,N_1,N_2+1)\cdot e^{-pV/kT}}{\Delta(T,p,N_1,N_2+1)} \quad (C.5)$$

$$\langle V\rangle_{N_2}=\frac{\sum_V V\cdot Q(T,V,N_1,N_2)\cdot e^{-pV/kT}}{\Delta(T,p,N_1,N_2)} \quad (C.6)$$

The above two average volumes are not identical; in fact their difference defines the molecular partial volume of the solute:

$$v_2=\langle V\rangle_{N_2+1}-\langle V\rangle_{N_2} \quad (C.7)$$

It is clear, therefore, that the definition of the chemical potential in the T,p,{N} ensemble includes a definite change in the average volume.

In the thermodynamic limit we are allowed to: 1) substitute the maximum term (corresponding to the most probable volume) for the whole partition function; 2) assume that the most probable volume and the average volume are equal. Accordingly, Eqs. (C.2) and (C.3) reduce to:

$$\Delta(T,p,N_1,N_2+1) \quad (C.8)$$
$$=Q(T,\langle V\rangle_{N_2+1},N_1,N_2+1)\cdot\exp(-p\langle V\rangle_{N_2+1}/kT)$$

$$\Delta(T,p,N_1,N_2) \quad (C.9)$$
$$=Q(T,\langle V\rangle_{N_2},N_1,N_2)\cdot\exp(-p\langle V\rangle_{N_2}/kT)$$

where it is understood that the average volumes must be expressed in terms of T,p,N_1 and N_2, in order to obtain the appropriate functional dependence of the Δ partition functions. Substitution of Eqs. (C.8) and (C.9) in Eq. (C.2) and use of Eq. (C.7) yields the following expression for the chemical potential in T,p,N ensemble:

$$\mu_2[T,p,\{N\}] \quad (C.10)$$
$$=-kT\cdot\ln\frac{Q(T,\langle V\rangle_{N_2+1},N_1,N_2+1)\cdot\exp(-pv_2/kT)}{Q(T,\langle V\rangle_{N_2},N_1,N_2)}.$$

The volume-dependence of the canonical partition function is given by $(\partial \ln Q/\partial V)=p/kT$, which can be integrated from $\langle V\rangle_{N_2}$ to $\langle V\rangle_{N_2+1}$ to yield:

$$Q(T,\langle V\rangle_{N_2+1},N_1,N_2+1) \quad (C.11)$$
$$=Q(T,\langle V\rangle_{N_2},N_1,N_2+1)\cdot\exp(pv_2/kT)$$

and substitution in C.10 leads to:

$$\mu_2[T,p,\{N\}]=-kT\cdot\ln\frac{Q(T,<V>_{N_2},N_1,N_2+1)}{Q(T,<V>_{N_2},N_1,N_2)} \quad (C.12)$$

which must be compared with Eq. (C.1) (and also with Eq. (C.2)). It should be clear from that comparison that the chemical potential in the T,V,{N} and T,p,{N} ensembles are identical in the thermodynamic limit, provided that the volume of the systems in the T,V,{N} ensemble is equal to the average volume of the T,p,{N} ensemble *prior to the addition of the molecule*. Of course, addition of the molecule will cause the average volume to change according to its molecular partial volume.

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