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Transfer Free Energies and Average Static Accessibilities for Ribonuclease A in Guanidinium Hydrochloride and Urea Solutions†

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ABSTRACT: Isopiestic vapor pressure measurements have been used to obtain free energies of transfer of ribonuclease A from dilute buffer to solutions of either urea or guanidine hydrochloride (GdnHCl) over a wide cosolute concentration range. The free energies of transfer vary monotonically from 0 to −8 kcal/mol in 8 M urea and to −18 kcal/mol in 6 M GdnHCl. These values are not large in relation to free energies of transfer of constituent groups of the protein from water to cosolute solutions of the same concentration. The assumption is made that the magnitude of the free energy of transfer of the protein is governed by the average static accessibility of its constituent groups to the solution. The free energies of transfer to different cosolute concentrations of a hypothetical 100% accessible ribonuclease A were determined using literature values of the free energies of transfer of constituent groups and the amino acid composition. The ratio of the experimentally determined free energy of transfer to the free energy of transfer of the 100% accessible protein gave 11% accessible surface area for the native protein in 1 M GdnHCl or 2 M urea. Additional considerations led to a value of 36% for the accessible surface area of the denatured protein in 6 M GdnHCl or 8 M urea.

Detailed consideration of solvation is of major importance in providing an accurate description of the conformation of a protein in solution. Only the preferential solvation, i.e., the preference of the protein for one or the other component of a mixed solvent, is completely defined by thermodynamic measurements. Numerical measures for it have been obtained in protein-cosolute-water systems (Noelken and Timasheff, 1967; Noelken, 1970; Span and Lapanje, 1973, Span et al., 1974) by a combination of equilibrium dialysis and differential refractometry and also (Hade and Tanford, 1967) by the use of isopiestic measurements. However, it has been difficult to utilize these values for further elaboration of protein conformation in solution (Tanford, 1970; Franks and Eagland, 1975).

The preferential solvation formalism conventionally adopted tends to obscure the fact that interactions between two solutes in aqueous solution can equally well be treated in terms of the free energies of transfer of the protein from dilute buffer to aqueous solutions of cosolutes of interest. Consideration of interactions in these terms has several advantages. For one thing, such free energy changes can readily be compared for different proteins and different cosolutes. In addition, comparison is also possible with the free energies of transfer of the constituent groups of the protein obtained in other experiments using small model compounds (Nozaki and Tanford, 1963, 1970).

In the work to be described, we have obtained free energies for the transfer of ribonuclease A from dilute buffer to solutions of urea and GdnHCl,[1] respectively, over wide cosolute concentration ranges. Combination of these results with previously obtained thermodynamic data (Nozaki and Tanford, 1963, 1970) allows an experimental estimate of the average static accessibility of the constitutent groups of both the native and unfolded protein to these solutions.

Experimental Section

Materials. Ribonuclease A was purchased from Sigma Chemical Co. While type IIA was used for most of the runs, the more extensively chromatographed type XIIA was also employed in a few runs to test the effect of ribonuclease A homogeneity on the results. Within experimental error, no difference was found between runs with the two types of protein. The protein was dialyzed at 2 °C against three changes of 0.1 M KCl and, subsequently, against three changes of doubly distilled water. The dialyzed solutions were passed through a mixed-bed ion-exchange column (Rohm and Haas Amberlite MB-1). The pH of the protein solution as it came off the column was found to be 9.6–9.7, in agreement with the value of 9.6 reported previously (Tanford and Hauenstein, 1956). The protein solutions were lyophilized at concentrations

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[1] Abbreviation used: GdnHCl, guanidine hydrochloride.

TABLE I: Average Solute Molalities in the Isopiestic Solutions (1) Urea + Water and (2) Urea (m_c) + Ribonuclease A (m_p) + Water and Values of $-\Delta/(m_c m_p)$ Calculated Therefrom at 25 °C.

Solution 1	Solution 2		
m_{urea}	m_c	m_p	$-\Delta/(m_c m_p)$
1.0226	1.0241	0.002374	1.57
2.1514	2.1540	0.001143	1.42
3.4720	3.4767	0.001256	1.18
4.0014	4.0087	0.001091	1.62
4.8608	4.8663	0.001363	0.87
5.5237	5.5301	0.001468	0.80
7.4427	7.4533	0.001397	0.91
8.0138	8.0246	0.001224	0.96
9.5000	9.5151	0.001580	0.86
11.0268	11.0504	0.001734	1.02
12.3356	12.3517	0.001760	0.63
13.7073	13.7226	0.001905	0.50
14.8833	14.9048	0.001785	0.66
14.9582	14.9825	0.001828	0.71

TABLE II: Average Solute Molalities in the Isopiestic Solutions (1) Sodium Chloride + Water and (2) GdnHCl(m_c) + Ribonuclease A(m_p) + Water and Values of $-\Delta/(m_c m_p)$ Calculated Therefrom at 25 °C.

Solution 1	Solution 2		
m_{NaCl}	m_c	m_p	$-\Delta/(m_c m_p)$
0.6793	0.7670	0.002312	5.40
1.2343	1.5154	0.001401	3.26
1.5649	2.0160	0.001153	3.09
2.0299	2.7858	0.001419	3.23
2.1768	3.0470	0.001505	4.30
2.6931	3.9961	0.001161	3.19
2.7778	4.1665	0.001504	3.67
2.9264	4.4599	0.001535	3.40
3.4498	5.5488	0.001653	3.92
3.6668	6.0006	0.001220	2.26
3.8805	6.4656	0.001045	1.74
4.2345	7.2721	0.001848	2.31
5.0150	9.1182	0.002065	1.85
5.8383	11.1827	0.001952	1.07

below 1% to avoid possible aggregation (Crestfield et al., 1963). Several runs were carried out with protein that was taken directly from the mixed-bed column and not lyophilized. No effect of lyophilization of ribonuclease A on the isopiestic results was noted within experimental error. Protein concentrations were established by conventional dry weight procedures.

Urea (Baker) was recrystallized twice from hot (<60 °C) methanol solutions. It was vacuum dried and stored over $MgClO_4$. GdnHCl (Eastman or Aldrich) was recrystallized using one of the published procedures (Nozaki and Tanford, 1967), i.e., a first recrystallization from a hot ethanol–benzene mixture and a second recrystallization from water at 40 °C with the use of a rotary evaporator to remove the solvent. The material was vacuum dried and stored over $MgClO_4$.

All solutions were made up using doubly distilled water. The molality scale was employed throughout—mol of solute/1000 g of water as solvent.

Method. The isopiestic apparatus described previously (Schrier and Robinson, 1970) was employed. As in the past, silver dishes were used to contain the NaCl reference solutions, the separate cosolute solutions, and the urea + ribonuclease A solutions. However, we found that the GdnHCl + ribonuclease A mixtures attacked the silver dishes. The problem was solved by inserting small glass or Teflon liners into the dishes. The modified dishes were checked in the usual way by measuring the isopiestic ratio for NaCl solutions against KCl solutions. While equilibration times increased slightly, there was no loss of precision or accuracy by the use of this modification. A few runs were made using urea + ribonuclease A solutions in these dishes with results not discernibly different from those obtained using the silver dishes alone.

The desiccator in a typical run contained nine dishes in contact with a large copper block. One group of three dishes contained a NaCl solution, a second group of three contained a solution of the cosolute (either urea or GdnHCl), while the last group of three dishes contained the protein + cosolute solution. For the systems involving urea, we were able to use the aqueous urea solutions as the isopiestic reference, since high quality osmotic coefficients are available (Ellerton and Dunlop, 1966). The NaCl solutions in the runs with urea + ribonuclease A served as a check on the establishment of vapor phase equilibrium in the overall system. For the purposes of this test, we sought good agreement of our experimentally determined osmotic coefficients for NaCl with literature values. The use of NaCl as the reference in the runs with GdnHCl + ribonuclease A allowed us to obtain osmotic coefficients for GdnHCl (Schrier and Schrier, 1976).

All equilibrations were carried out at 25.0 ± 0.1 °C. As a further check on the establishment of vapor phase equilibrium, a variable number of drops of water were added to two of the three dishes of a particular set so that, if equilibration were not achieved during a run, the resulting molalities of the individual solutions would be imprecise. This check and the test mentioned above allowed us to establish that 6 days sufficed to achieve vapor phase equilibrium in all runs in which the cosolute concentration was greater than 2 m. The more dilute systems required 10 days–2 weeks.

Results

The results of a given run may be conveniently expressed in terms of three average molalities, that of the reference substance in the dishes containing the reference solution alone and the average molalities of cosolute and ribonuclease A, respectively, in the dishes containing the cosolute plus protein solution. These average molalities are given in Table I for the runs with urea and in Table II for those runs with GdnHCl. For a given run, the average molalities are related in that they are solute concentrations of solutions which are in isopiestic equilibrium.

These data can be treated using a scheme (Robinson and Stokes, 1961) that makes use of the Gibbs–Duhem equation and yields free energies of transfer of one solute from water to solutions of the other solute. A function, Δ, is defined as:

$$\Delta = -55.51[\ln a_w(\text{ref}) - \ln a_w(\text{cosolute}) - \ln a_w(\text{ribonuclease A})] \quad (1)$$

In this equation, a_w(ref) is the water activity of the reference solution, which is urea for the urea + ribonuclease A runs, and NaCl for those involving GdnHCl + ribonuclease A. We note that the water activity of the cosolute + ribonuclease A solution is equal to a_w(ref) at isopiestic equilibrium. The quantity, a_w(cosolute), is the activity of water in a cosolute solution which has the same concentration of cosolute as the mixture of cosolute + ribonuclease A. Similarly, a_w(ribonuclease A) is the activity of water in a ribonuclease A solution having the same concentration of ribonuclease A as the mixture. We want

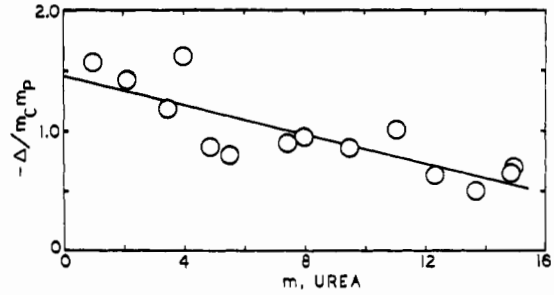

FIGURE 1: The dependence of the quantity, $\Delta/(m_c m_p)$, for ribonuclease A on urea molality at 25 °C.

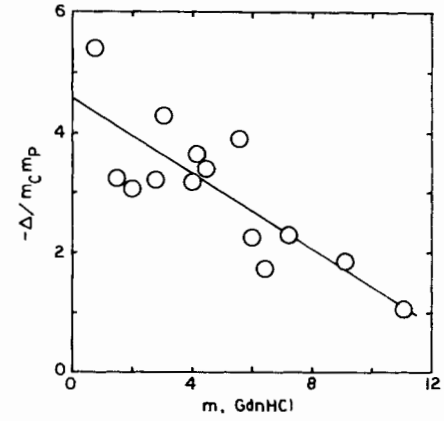

FIGURE 2: The dependence of the quantity, $\Delta/(m_c m_p)$, for ribonuclease A on GdnHCl molality at 25 °C.

to emphasize that a_w(cosolute) and a_w(ribonuclease A) refer to solutions in which there is a single solute. The function, Δ, thus measures the difference between the contribution of the mixed solutes to establishing the activity of water in the system and the sum of the contribution of the solutes taken separately. A nonzero value of Δ is attained if the solutes interact in some way with each other in the mixed solution rather than behaving just as they did in their separate solutions. The value of Δ is negative if this interaction is favorable.

For purposes of evaluation eq 1 may be rewritten as:

$$\Delta = \upsilon_{ref} m_{ref} \phi_{ref} - \upsilon_c m_c \phi_c^\circ - m_p \phi_p^\circ \qquad (2)$$

In eq 2, m_{ref} is the molality of the reference solution and ϕ_{ref} is the osmotic coefficient of the reference at that molality. The quantity υ_{ref} is equal to 1 for urea and 2 for NaCl. In the term, $\upsilon_c m_c \phi_c^\circ$, υ_c is also an integer, 1 for urea and 2 for GdnHCl, m_c is the cosolute molality, and ϕ_c° is the osmotic coefficient at the molality of cosolute in the separate solution. Osmotic coefficients for sodium chloride are available (Robinson and Stokes, 1968), while the sources of these quantities for urea and GdnHCl have been mentioned above.

The final term in eq 2 measures the contribution of the protein itself to the establishment of the water activity. Since the protein molality is quite small in these experiments, generally around $0.0015m$, the term is small but it is not negligible. The osmotic coefficient of the protein in water has not been measured, but a good estimate can be made as follows. Lapanje and Tanford (1967) measured the osmotic properties of ribonuclease A in 6 M GdnHCl. From their data, we have derived an equation for the osmotic coefficient of ribonuclease A in this solvent, $\phi = 1 + 0.0161 C_p$, where C_p is the concentration of the protein in grams per liter. In a related study, Castellino and Barker (1968) have measured the second virial coefficients of a number of proteins in both dilute buffer and 6 M GdnHCl. From their data, the second virial coefficient of proteins is about 30 times larger in 6 M GdnHCl than in dilute buffer. Using this figure and converting to molal units to express the protein concentration, we obtain $\phi = 1 + 7.34 m_p$ for the osmotic coefficient of ribonuclease A in dilute buffer. This equation can now be utilized in the evaluation of Δ.

Values of Δ were calculated for each set of molalities using eq 2. These values are given as $\Delta/(m_c m_p)$ in Tables I and II. This is the most useful way to express the function since $\Delta/(m_c m_p)$ values are directly related to free energies of transfer. The results are also shown as a function of cosolute molality in Figures 1 and 2.

Runs at similar cosolute molalities with different protein molalities indicate that the values of $\Delta/(m_c m_p)$ are independent of protein molality within experimental error as expected at the low protein molalities utilized here. This not only allows the calculation of free energies of transfer (see below) but also enables us to consider the derived free energies of transfer as infinite dilution values.

After examining several functional forms, the values of $\Delta/(m_c m_p)$ were fit to the following equation

$$\Delta/(m_c m_p) = a + b m_c \qquad (3)$$

using a least-squares routine. The lines shown in Figures 1 and 2 were calculated with eq 3. The values of the parameters and their standard deviations based on the least-squares fit are as follows: for urea + ribonuclease A, $a = -1.465 \pm 0.076$, $b = 0.0601 \pm 0.0082$; for GdnHCl + ribonuclease A, $a = -4.582 \pm 0.243$, $b = 0.314 \pm 0.043$. The overall standard deviation of the fit was 21% for the urea + ribonuclease A runs and 22% for those involving GdnHCl + ribonuclease A.

Since the entire experimental procedure depends on weighing and handling the dishes, as well as whether isopiestic equilibrium is achieved, we expect the one major source of uncertainty to be related to the precision of the derived molalities of the various solutes. The usual standard deviation of the mean of these molalities was on the order of $\pm 0.04\%$. Because the values of Δ are so small, particularly in the urea + ribonuclease A systems, uncertainties of the above magnitude produced estimated maximum uncertainties of $\pm 26\%$ in the final value of $\Delta/(m_c m_p)$ in the urea + ribonuclease A systems and $\pm 18\%$ in the GdnHCl + ribonuclease A systems. These values are similar to the statistical measure of overall fit given above, implying the absence of systematic error, e.g., deviations from equilibrium.

The relation between $\Delta/(m_c m_p)$ and the free energy of transfer follows directly from the treatment of Robinson and Stokes (1961). The following relation can be shown:

$$\Delta/(m_c m_p) = (\partial \ln \gamma_p / \partial m_c)_{m_p} \qquad (4)$$

where γ_p is the molal activity coefficient of the protein at a particular cosolute molality. Combining eq 3 and 4 and integrating at constant protein molality give

$$\ln \gamma_p/\gamma_p^\circ = a m_c + \tfrac{1}{2} b m_c^2 \qquad (5)$$

In eq 5, γ_p° is the molal activity coefficient of the protein in dilute buffer. Finally:

$$\Delta G_{tr} = RT \ln \gamma_p/\gamma_p^\circ \qquad (6)$$

where 1 mol of protein is transferred from dilute buffer to the cosolute solution of interest.

The values of ΔG_{tr} on the molal scale for the transfer of ribonuclease A from dilute buffer to the various cosolute concentrations are shown in Figure 3a for urea and in Figure 3b

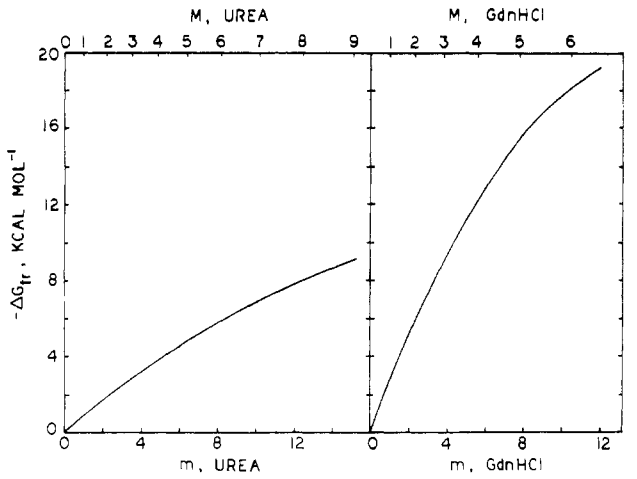

FIGURE 3: (a) The molal free energies of transfer of ribonuclease A from dilute buffer to urea solutions of various molalities (lower scale) or molarities (upper sclae) at 25 °C. (b) The molal free energies of transfer of ribonuclease A from dilute buffer to GdnHCl solutions of various molalities (lower scale) or molarities (upper scale) at 25 °C.

for GdnHCl. For convenience in our further discussion, both molality and molarity scales are shown for each cosolute. The density data given by Egan and Luff (1966) were used to obtain urea molarities, while those for GdnHCl were obtained using the data of Kielly and Harrington (1960) with correction to 25 °C.

Discussion

The results presented in Figure 3 have the expected negative sign. Additionally, the favorable (relative to water) solvation of ribonuclease A in GdnHCl solutions is greater than that for urea. What is unexpected is that the transfer free energies of ribonuclease A are not really large in proportion to the same quantities for the transfer of individual constituent groups of the protein from water to denaturant solutions of the same concentration. For instance, the free energy of transfer of a mole of tyrosine side chains, $-CH_2C_6H_4$-*p*-OH, from water to 2 M urea is −0.23 kcal/mol (Nozaki and Tanford, 1963), while the free energy of transfer of 1 mol of ribonuclease A from water to 2 M urea is −1.8 kcal/mol, a number only eight times as large.

This result can be interpreted in either of two ways. On the one hand, it is possible that the contributions of individual groups on the protein to the overall free energy of transfer are highly nonadditive. Although there have been no studies of additivity of constituent group properties with a series of large molecules, work with small molecules and their oligomers (Schrier and Schrier, 1967; Nandi and Robinson, 1972) suggests the additivity of constituent group free energies of transfer to a good first approximation. In addition, the consistency of estimates of $\Delta G_U°(H_2O)$ of various proteins using different methods (Pace, 1975), one of which involves the assumption of additivity of free energy contributions of amino acid side chains and peptide backbone units, suggests that additivity of constituent group effects is preserved in accounting for the thermodynamic properties of macromolecules.

Another interpretation is that groups which are not accessible to the solvent medium because of their position in the interior of the protein will not contribute to its free energy of transfer. This notion is consistent with the concepts of Tanford (1970) regarding protein denaturation, i.e., that groups which are buried in the native protein become exposed to the solvent as the protein is unfolded. If the proposal has validity, we would expect the free energy of transfer of the protein to increase more rapidly with respect to cosolute concentration than does the free energy of transfer of a constituent group over the same concentration range. That this is the case can be seen by considering the free energy of transfer of the peptide backbone unit to 1 and 6 M GdnHCl (Pace, 1975). The value at 6 M is 2.5 times the value at 1 M. In contrast, the free energies of transfer of ribonuclease A from water to 1 and 6 M GdnHCl solutions given in Figure 3b are −2.8 and −18.3 kcal/mol, nearly a sevenfold difference.

We suggest, therefore, that the free energy of transfer of ribonuclease A from water to solutions of cosolutes can be correlated to the average accessibility of groups on the protein to the external medium, i.e., the solute-solvent combination. To utilize these values in a quantitative measure of accessibility, some method of defining 100% accessible surface area is required. The available data (Pace, 1975) obtained for the transfer of side chains and backbone peptide units from water to urea or GdnHCl solutions permit the following definition of the free energy of transfer of a 100% accessible protein from water to a cosolute solution of a particular concentration:

$$\Delta G_{tr,100\%} = \sum_i n_i \Delta g_{tr,i} \qquad (7)$$

where n_i is either the number of amino acid side chains of a particular class or the number of peptide backbone units in the protein, and $\Delta g_{tr,i}$ is the free energy of transfer of 1 mol of a given side chain or peptide backbone unit from water to a cosolute solution at a particular concentration. It should be noted that the value of $\Delta G_{tr,100\%}$ is independent of the concentration units employed to denote cosolute concentration, since the $\Delta g_{tr,i}$ values are obtained by difference between the ΔG_{tr} of the amino acid in question and that for glycine.

A listing of a number of values of $\Delta g_{tr,i}$ collected from different sources is given in the review by Pace (1975). Since previous work was concerned with the *difference* in the free energies of transfer between the native and the unfolded proteins, free energy of transfer values for those amino acid side chains which were assumed to be exposed to the medium in both the native and unfolded states were not determined. We have used various assumptions to provide values for Δg_{tr} for the side chains of aspartic acid, arginine, glutamic acid, lysine, and serine. Multiplication of the $\Delta g_{tr,i}$ values at 2 and 8 M urea and at 1 and 6 M GdnHCl by the number of the *i*th side chain in ribonuclease A or by the number of peptide backbone units leads to values for the different contributions which can be summed to give $\Delta G_{tr,100\%}$. The values are given in Table III.

One more point must be considered before accessibilities can be calculated. The protein changes completely from the native to the unfolded conformation when it is transferred from dilute buffer to cosolute concentrations equal to or exceeding 3.5 M GdnHCl or 8.0 M urea. This fact may be utilized to ascertain the accessibility of groups of the unfolded protein independent of any assumptions about the accessibility of groups of the native protein.

Let us assume that the two-state model of unfolding is valid for urea- or GdnHCl-induced isothermal unfolding of ribonuclease A at 25 °C. The thermodynamic cycle which Tanford (1970) applied to this process is shown in Figure 4. The left hand vertical arrow denoted by (i) corresponds to transfer of the protein from dilute buffer to any cosolute solutions below the onset of the unfolding transition. The experimental free energy of transfer values (Figure 3), below unfolding, corresponds to process i. Process iii in Figure 4 does not have such a direct relationship to experimentally measured quantities,

TABLE III: Free Energies for the Transfer of the Constituent Groups of Ribonuclease A from Water to Aqueous Cosolute Solutions at 25 °C.

	ΔG_{tr}(kcal/mol)			
	Urea		GdnHCl	
	2 M	8 M	1 M	6 M
Peptide	−6.03	−15.99	−10.21	−30.14
Ala	0.0	0.12	−0.12	−0.54
Arg	−0.40	−1.02	−0.72	−1.68
Asn	−1.49	−4.73	−2.20	−7.10
Asp	−0.54	−1.72	−0.80	−2.58
½-Cys	−0.92	−3.32	−1.20	−4.28
Gln	−0.48	−1.38	−0.81	−2.16
Glu	−0.48	−1.38	−0.81	−2.16
Gly	0.0	0.0	0.0	0.0
His	−0.40	−1.02	−0.72	−1.68
Ile	−0.30	−0.80	−0.41	−1.29
Leu	−0.22	−0.59	−0.30	−0.96
Lys	−1.00	−2.65	−1.35	−4.30
Met	−0.46	−1.66	−0.60	−2.14
Phe	−0.54	−1.80	−0.65	−2.33
Pro	−0.30	−0.80	−0.40	−1.28
Ser	0.0	0.15	−0.15	−0.68
Thr	−0.40	−1.15	−0.65	−1.25
Tyr	−1.35	−4.41	−1.41	−4.62
Val	−0.54	−1.44	−0.77	−2.39
Total	−15.85	−45.59	−24.28	−73.56

TABLE IV: Average Static Accessibilities of Constituent Groups of Ribonuclease A to Solutions of Urea or GdnHCl at 25 °C.

Urea		GdnHCl	
2 M	8 M	1 M	6 M
11.4 ± 2.4	34.4 ± 4.3	11.5 ± 2.5	37.2 ± 5.6

however. Instead, the measured free energies of transfer of ribonuclease A from dilute buffer to cosolute concentrations exceeding 4 M GdnHCl or 8 M urea corresponds to process v, which is denoted by the dotted diagonal line. In order to obtain free energy values for process iii, i.e., the transfer of *unfolded* ribonuclease A from dilute buffer to cosolute solutions, we must have values for process iv. Fortunately, a careful study of the ribonuclease A transition in GdnHCl and urea solutions has recently been made (Green and Pace, 1974). The following values for process iv were obtained by linear extrapolation of data from the unfolding region: $\Delta G_U°(H_2O)$ = 9.3 kcal/mol for GdnHCl solutions and $\Delta G_U°(H_2O)$ = 7.7 kcal/mol for urea solutions. The possible reasons for the differences in these values are discussed by Pace (1975). We will retain the separate values in further calculation rather than averaging them.

With these values on hand, it follows from Figure 4 that

$$\Delta G_{iii} = \Delta G_v - \Delta G_{iv} \quad (8)$$

For the transfer of unfolded ribonuclease A from dilute buffer to 6 M GdnHCl we obtain $\Delta G_{tr} = -27.6 \pm 6.1$ kcal/mol, while for the transfer to 8 M urea $\Delta G_{tr} = -15.8 \pm 3.3$ kcal/mol.

We can now calculate the accessibility of native and unfolded ribonuclease A using the relation:

$$\text{accessibility} = (\Delta G_{tr}/\Delta G_{tr,100\%})100 \quad (9)$$

The accessibility values obtained using transfer free energies from dilute buffer to 1 and 6 M GdnHCl and from dilute

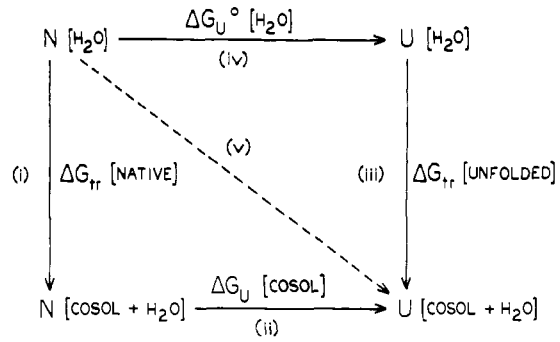

FIGURE 4: The thermodynamic cycle for the isothermal unfolding of ribonuclease A by added cosolute. For an explanation of the symbols, see text. After Tanford (1970).

buffer to 2 and 8 M urea are given in Table IV. In calculating these quantities, we have removed cratic contributions to ΔG_{tr} (Tanford, 1970). These amounted to no more than 1% of ΔG_{tr} in any case. The uncertainties given are those calculated from the ΔG_{tr} values above. The additional uncertainties in $\Delta G_{tr,100\%}$ have been neglected since they are impossible to estimate.

The values obtained for the accessibility of the native conformation in GdnHCl and urea solutions are nearly equal. A similar situation is encountered for the unfolded form. While the result may be fortuitous for the native conformation, Tanford (1968) has suggested that the conformation of ribonuclease A is the same in 6 M GdnHCl and in 8 M urea. We therefore expect similar accessibilities in the two solvents. This agreement between the accessibility values lends support to the experimental approach and assumptions used.

In order to compare these results with the accessibility values calculated for ribonuclease S by Lee and Richards (1971), we must assure ourselves that accessibility in their definition is at least qualitatively similar to accessibility as understood here. While their method determines the fraction of the surface area which is exposed to the solvent, the measure proposed in this paper is concerned with a ratio involving various free energies of transfer.

Fortunately, there have been several recent efforts which offer convincing evidence that a good correlation exists between the free energies of transfer of nonpolar submolecular units from one medium to another and the surface area of the units involved. For example, Hermann (1972) and Reynolds et al. (1974) have shown that there is a linear correlation between the solubility of hydrocarbons in water, i.e., the free energies of transfer between pure hydrocarbon and water, and the surface area of a hydrocarbon molecule calculated by packing spheres representing water molecules around the constitutent alkyl groups.

While the assumption of a similar relationship for the nonpolar groups of proteins and for the polar residues is more tenuous, there are implications of its existence, as, for example, in the Friedman cosphere theory (Friedman, 1973). Making the assumption that free energies of transfer of both polar and nonpolar groups from water to solutions of either cosolute are directly proportional to the surface area of the groups, suppose that the free energy of transfer of a protein, ΔG_{tr}, is proportional to its accessible surface area, A, with a proportionality constant, β,

$$\Delta G_{tr} = \beta A \quad (10)$$

As well, the sum of the free energies of transfer of the constituent groups is related to the total surface area of the groups

as defined in terms of the placement of water molecules around the groups. The proportionality constant is the same as in eq 10 so that

$$\Delta G_{tr,100\%} = \beta \sum a_i \quad (11)$$

Dividing eq 10 by eq 11 and multiplying by 100 give:

$$\text{accessibility} = (\Delta G_{tr}/\Delta G_{tr,100\%})100 = (A/\sum a_i)100 \quad (12)$$

Thus, our measure of accessibility and that given by Lee and Richards (1971) are commensurate.

The accessible surface area of native ribonuclease A in 1 M GdnHCl or 2 M urea, 11%, may be compared to the average accessible surface area calculated by Lee and Richards for ribonuclease S, 6%. The great similarity between the structures of the protein and the derivative rules out any difference there as the source of the discrepancy. The difference may actually be within the combined uncertainties of the two estimates. However, we must consider the fact that the ribonuclease S crystal is bathed by a salt solution which is obviously a poor solvent for the molecule, while the accessibility values reported here for the native protein are based on measurements made in the good solvents, 1 M GdnHCl or 2 M urea. Since the direction of change is necessarily toward greater accessibility relative to pure water in good solvents and is the reverse in poor solvents, the difference between the accessibility value reported by Lee and Richards and that reported here is understandable.

The discrepancy between the values of the accessibility for the unfolded protein reported here and that obtained by Lee and Richards from their calculations with amino acid models is large. Their value of 17% is far away from the 36% reported here. Furthermore, their model pertains to a protein devoid of cross-links, while the value we report is for ribonuclease A with cross-links in place. It should be noted, however, that their value was presented as a crude estimate and does not have the same significance as the accessibility calculated from the x-ray data for ribonuclease S.

Further work is required to generalize the accessibility results reported here. Both the free energies of transfer and the accessibilities will clearly be of value in the further delineation of protein conformational topology and energetics.

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