Nozaki, Y. (1970) *Methods Enzymol. 26*, 43–50.
Nozaki, Y., & Tanford, C. (1970) *J. Biol. Chem. 245*, 1648–1652.
Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1–43.
Pickover, C. A., McKay, D. B., Engelman, D. M., & Steitz, T. A. (1979) *J. Biol. Chem. 254*, 11323–11329.
Privalov, P. L. (1982) *Adv. Protein Chem. 35*, 1–104.
Robson, B., & Pain, R. H. (1973) *Jerusalem Symp. Quantum Chem. Biochem. 5*, 161–172.
Rowe, E. S., & Tanford, C. (1973) *Biochemistry 12*, 4822–4827.
Schellman, J. A. (1975) *Biopolymers 14*, 999–1018.
Schellman, J. A. (1978) *Biopolymers 17*, 1305–1322.
Schellman, J. A., & Hawkes, R. B. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 331–343, Elsevier/North-Holland Biochemical Press, Amsterdam.
Scopes, R. K. (1969) *Biochem. J. 113*, 551–554.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Teale, J. M., & Benjamin, D. C. (1976a) *J. Biol. Chem. 251*, 4603–4608.
Teale, J. M., & Benjamin, D. C. (1976b) *J. Biol. Chem. 251*, 4609–4615.
Teale, J. M., & Benjamin, D. C. (1977) *J. Biol. Chem. 252*, 4521–4526.
Warren, J. R., & Gordon, J. A. (1966) *J. Phys. Chem. 70*, 297–300.
Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697–701.
Wetlaufer, D. B. (1981) *Adv. Protein Chem. 34*, 61–92.
Wong, K. P., & Tanford, C. (1973) *J. Biol. Chem. 248*, 8519–8523.
Zêtina, C. R., & Goldberg, M. E. (1980a) *J. Mol. Biol. 137*, 401–414.
Zêtina, C. R., & Goldberg, M. E. (1980b) *J. Biol. Chem. 255*, 4381–4385.

Effects of Urea and Guanidine Hydrochloride on Peptide and Nonpolar Groups†

Pradip K. Nandi and Dwight R. Robinson*

ABSTRACT: The free energy transfer of several *N*-acetyl(glycine)$_n$ ethyl esters (n = 1–3) and side chain derivatives (Ala, Val, Nva, Leu, Nle, and Phe) from water to urea and guanidine hydrochloride solutions has been determined from the solubility and distribution coefficients of these compounds between aqueous and nonaqueous phases. These uncharged model peptides, unlike the amino acids used for a similar study, avoid complication due to charge effects for the transfer process. The compounds with an increase in the number of glycyl groups show additivity of the group free energy toward the transfer from water to urea solution but not to guanidine hydrochloride solution. The derivatives with a side chain show that the principle of group additivity does not hold true for the aliphatic side chains for the transfer to either urea or guanidine hydrochloride solutions. In fact, the free energy of transfer of the side chains, viz., aliphatic ones, is found to be energetically unfavorable in moderately high denaturant concentration. Phenylalanyl, the only aromatic side chain studied here, showed a favorable free energy of transfer to the denaturant solutions. In addition, the values of the favorable free energy obtained in this study are much smaller than the values obtained from the study of the amino acids. The transfer of the glycyl group to the denaturant solutions is exothermic whereas the transfer of the side chains is endothermic in nature. The interaction of these solutes with urea and guanidine hydrochloride solutions has been explained on the basis of the hypothesis of Roseman & Jencks [Roseman, M., & Jencks, W. P. (1975) *J. Am. Chem. Soc. 97*, 631–640], which states that the driving force for the favorable interaction effect of cosolvents with solutes arises from a more favorable sum of the free energies of cavity formation and nonpolar interaction in the presence of cosolvents (denaturants). With polar solutes, the cosolvent must also be able to form hydrogen bonds to make the overall free energy of transfer favorable.

A number of studies have been made of the interaction of denaturants urea and Gdn·HCl[1] with model compounds to understand the mechanism of denaturation of proteins in these solutions (Tanford, 1968, 1970; Pace, 1975; Eagland, 1975; Franks & Eagland, 1976). Small molecules, viz., amino acids and peptides, have been used mostly to understand the thermodynamic nature of interaction of urea and Gdn·HCl with the amino acid residues in proteins. The results have been generally obtained from solubility and calorimetric measurements (Nozaki & Tanford, 1963, 1970; Robinson & Jencks, 1965a; Wetlaufer et al., 1964; Lapanje et al., 1978; Schonert & Stroth, 1981; Cesaro et al., 1982). From the solubility studies of a large number of amino acids in urea and Gdn·HCl, Tanford and co-workers (Nozaki & Tanford, 1963, 1970; Tanford, 1970) observed that the free energies of transfer, Δg_{tr}, of the contributing groups in the amino acids from water to denaturant solutions are additive and independent of the nature of the molecule (Wetlaufer et al., 1964).[2] The Δg_{tr} values for individual groups can then be used to calculate the stability of the protein molecule in denaturant solution from its amino

† From the Clinical Endocrinology Branch, NIADDK, National Institutes of Health, Bethesda, Maryland 20814 (P.K.N.), and the Arthritis Unit, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02114 (D.R.R.). *Received May 1, 1984.* Supported by grants from the NIADDK and the National and Massachusetts Chapters of the Arthritis Foundation.

[1] Abbreviations: Ag_1E, Ag_2E, Ag_3E, and Ag_4E, acetylmonoglycine, -diglycine, -triglycine, and -tetraglycine ethyl esters, respectively; AAE, AVE, AnVE, ALE, AnLE, and APE, acetylalanine, -valine, -norvaline, -leucine, -norleucine, and -phenylalanine ethyl esters; Gdn·HCl, guanidine hydrochloride.

[2] The principal of additivity is based on the consideration that the free energies of transfer of the constituent groups in the compound would yield the total free energy of transfer of the compound.

0006-2960/84/0423-6661$01.50/0

Table I: Distribution Coefficients and Solubilities of the Amino Acid Ethyl Esters[a]

compd	ref phase	temperature 0 °C	25 °C	40 °C
Ag_1E	chloroform	1.16 ± 0.01 (5)	0.814 ± 0.009 (15)	0.716 ± 0.006 (7)
Ag_2E	chloroform	35.37 ± 0.40 (5)	19.15 ± 0.38 (19)	14.44 ± 0.22 (8)
Ag_3E	solubility	2.43 ± 0.01 (5)	6.48 ± 0.006 (8)	11.08 ± 0.45 (5)
AAE	chloroform	0.373 ± 0.004 (5)	0.230 ± 0.007 (10)	0.193 ± 0.003 (7)
AnVE	butyl ether	13.79 ± 0.09 (5)		2.29 ± 0.03 (6)
	petroleum ether		20.24 ± 0.19 (7)	
AVE	petroleum ether		19.08 ± 0.03 (7)	
AnLE	butyl ether	3.54 ± 0.04 (5)		0.600 ± 0.006 (6)
	petroleum ether		4.75 ± 0.026 (8)	
ALE	butyl ether		1.88 ± 0.04 (7)	0.800 ± 0.005 (5)
	petroleum ether		7.27 ± 0.12 (9)	
APE	solubility	2.59 ± 0.06 (5)	3.41 ± 0.08 (13)	6.62 ± 0.10 (5)

[a] The values of the distribution coefficients represent the ratio of the concentrations of the compounds in water and in the reference phase. Solubilities are expressed in g/L. Values of the distribution coefficients and solubilities are followed by the standard error of determination and figures in parentheses denote the number of determinations.

for individual groups can then be used to calculate the stability of the protein molecule in denaturant solution from its amino acid composition. Pace (1975) observed that the additivity of free energy contributions of amino acid side chains and peptide backbone units can account for the thermodynamic properties of the protein molecule in denaturant solutions. The relatively smaller value of the free energy of transfer of ribonuclease A from water to the denaturant solutions compared to free energy values obtained from the additivity of the constituent groups (based on model amino acid transfer values) led Schrier and co-workers (Schrier & Schrier, 1976; Almog et al., 1978) to determine the average static accessibility of the peptide groups of the protein in solutions.

Robinson & Jencks (1965a,b) studied the solubility of acetyltetraglycine ethyl ester (Ag_4E) in urea, Gdn·HCl, and concentrated electrolyte solutions to understand the mechanism of protein denaturation in these solutions. By varying the number of the peptide[3] groups (CH_2CONH) from one to three in acetylglycine esters and comparing the results obtained by Robinson & Jencks (1965b) for the tetraglycine derivative, Ag_4E, we found the additive nature of Δg_{tr} values for the CH_2CONH group for the transfer from water to concentrated electrolyte solutions (Nandi & Robinson, 1972a). In addition, by use of these blocked amino acid derivatives, the Δg_{tr} values of the side chains, viz., alanine, valine, norvaline, leucine, norleucine, and phenylalanine, were also found to be additive for the transfer from water to electrolyte solutions (Nandi & Robinson, 1972b), and the results have been useful for the interpretation of properties of various proteins in salt solutions (Franks & Eagland, 1975; Eagland, 1975; Formisano et al., 1978; Poillon & Bertles, 1979; Herskovits et al., 1981). A serious lack of additivity of the Δg_{tr} values for the aliphatic and aromatic side chains, however, was observed for the transfer from water to tetraalkylammonium salt solutions (Nandi, 1974).

In this communication, we report the studies on the free energy of transfer of (i) the peptide (CH_2CONH) group from acetylglycine ethyl esters by varying the number of peptide groups from one to three and (ii) various side chains from water to urea and Gdn·HCl solutions. A serious lack of additivity of Δg_{tr} values is observed (i) for the transfer of peptide group to Gdn·HCl and (ii) for the transfer of the side chains to both urea and Gdn·HCl solutions.

Materials and Methods

[^{14}C]-*N*-Acetylglycine, -diglycine, -triglycine, -L-alanine, -L-valine, -L-norvaline, -L-leucine, and -L-norleucine ethyl esters were prepared by the method of Wolf & Nieman (1963). Details of their preparation, elemental analysis, isotopic purity, and characteristics have already been reported (Nandi & Robinson, 1972a,b). Acetyl-L-phenylalanine ethyl ester (grade I) from Cyclo Chemical Corp. was recrystallized from water before use. A solubility phase curve showed the absence of any impurity (Nandi, 1974). Urea and guanidine hydrochloride were reagent-grade chemicals from Mann. Glass-distilled water was used for all the experiments.

Radioactivity measurements were obtained with a Packard Model 3375 liquid scintillation spectrometer at 4 °C. Samples (0.1 mL) were added to the vials containing 10 mL of scintillation solution, the composition of which has already been described. The samples were counted for sufficient time to accumulate 20000 counts, in most cases reducing the standard errors of counting to 0.5% (Nandi & Robinson, 1972a,b; Nandi, 1974).

The solutes were equilibrated with solvents in 12-mL capacity tubes sealed with Teflon-lined screw caps, in both distribution and solubility measurements. The tubes were submerged in a water bath in a rotating rack, and mixing was accomplished by rotating tubes end over end and at 25–30 rpm. Temperatures were maintained at 0 ± 0.1, 25.0 ± 0.05, and 40.0 ± 0.1 °C.

Measurements of solubility with Ag_3E and APE at three temperatures and distribution of other esters and amides between the aqueous and reference phase at 25 and 40 °C were carried out by methods described in detail elsewhere (Nandi & Robinson, 1972a,b). Equilibration for solubility experiments was carried out for 10 days at 0 °C, 3 days at 25 °C, and 2 days at 40 °C. The equilibration time for distribution measurements was 1 h at both 25 and 40 °C and 5 h at 0 °C. That the above periods of equilibration were adequate for equilibrium to be attained was determined as described previously. Samples were removed for counting radioactivity from both phases of each tube to measure the concentration.

The concentration of APE was determined spectrophotometrically in a PMQ II Zeiss spectrophotometer, from the difference of absorption at 257.5 (λ_{max}) and 300 nm where the absorbance was small. Samples of the saturated solution were

[2] The principal of additivity is based on the consideration that the free energies of transfer of the constituent groups in the compound would yield the total free energy of transfer of the compound.

[3] The peptide group has been defined here by the formula CH_2CONH or $NHCH_2CO$, which represents the difference in stoichiometry between the successive members of the series of four peptide esters considered here. The peptide group, as defined here, contains one more hydrogen atom than the repeating peptide unit in protein (Nandi & Robinson, 1972a).

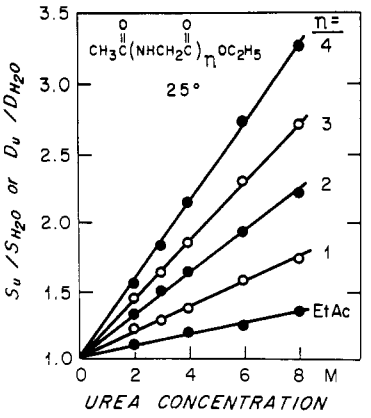

FIGURE 1: Effect of urea on the solubility of acetylglycine ethyl esters. The values of ethyl acetate and the tetraglycine derivative are from Robinson & Jencks (1965a). S_U and S_{H_2O} are solubilities in urea and water, respectively. D_U and D_{H_2O} are respectively the distribution coefficients in the presence and absence of urea.

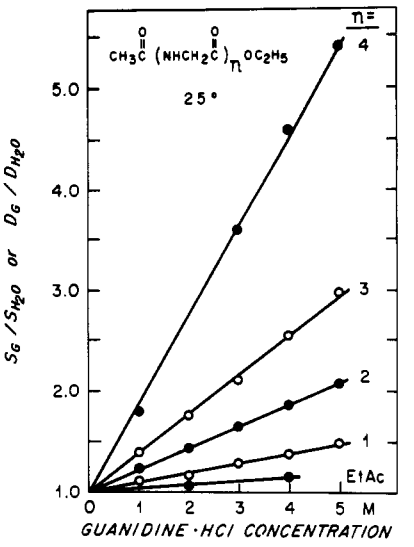

FIGURE 2: Effect of guanidine hydrochloride on the solubility of acetylglycine ethyl esters. The values of ethyl acetate and the tetraglycine derivative are from Robinson & Jencks (1965a). S_G, solubility in Gdn·HCl; D_G, distribution coefficient in Gdn·HCl.

diluted by factors of 6–30 with water for absorbance measurements. This reduces the denaturant concentration to levels where no significant effects on the UV spectra (if any) would be expected.

The precision of determination of solubility and distribution coefficients of the compounds employed here by utilizing similar techniques was previously described for all three temperatures (Nandi & Robinson, 1972a,b). In Table I, the standard deviations for the distribution and solubility measurements have been shown. We assume that the measurements in urea and guanidine hydrochloride solutions are of similar precision.

The concentrations of the compounds in the aqueous phase (in the presence of the denaturants) in the distribution experiments were 10^{-2} M, comparable to the concentration ranges of Ag_3E and APE obtained in the solubility experiments in denaturant solutions. A few of the experiments with APE in urea solutions using the distribution method (reference phase, petroleum ether) showed identical results as those obtained from solubility experiments. In addition, a 10-fold decrease in the concentrations of either Ag_2E or AnVE in distribution experiments did not show any effect on the distribution coefficients in urea solutions.

To test for mutual solubility of the phases in distribution experiments, 40-mL volumes of 8 M urea and 5 M guanidine hydrochloride were mixed with equal volumes of chloroform at 25 °C for 2 h. The phases were separated and solvent was removed from nonaqueous phase in vacuo. The residue was dried and weighed and was found to be sufficiently small (<0.01 M in the organic phase).

Results and Discussion

The values of the distribution coefficients (D_{H_2O}) reported here (Table I) are the ratio of the concentrations of the compounds in the aqueous phase to that in the reference phase. Solubility results for Ag_3E and APE in water have also been reported in the table. Although most of these values were reported previously (Nandi & Robinson, 1972a,b; Nandi, 1976), they are reproduced here since the effects of urea and Gdn·HCl have been reported in the present work as the ratio of either the distribution coefficient or the solubility in these solvents to that in water.

Effect on Ag_nE. The effects of increasing urea or Gdn·HCl on either the distribution or solubility values at 25 °C are shown graphically in Figures 1 and 2, with similar data for Ag_4E and ethyl acetate from the literature (Robinson & Jencks, 1965a). These five compounds differ from each other by the number of peptide groups ($-CH_2CONH-$) that they contain, and comparison of these compounds should provide a measure of the effects of urea and guanidine hydrochloride on the peptide group. There is a linear increase in solubility or distribution coefficients with urea and guanidine hydrochloride concentrations over their entire concentration range. Furthermore, the effects of both urea and guanidine hydrochloride increase with an increase in the number of peptide groups. Similar linear plots of the increased solubility were obtained for experiments at 0 and 40 °C but are not shown here. The effects of both urea and guanidine hydrochloride increase with decreasing temperature. This may indicate the polar nature of interaction of both the denaturant molecules with these compounds (Robinson & Jencks, 1965a; Roseman & Jencks, 1975).

The free energy of transfer, ΔG_{tr},[4] from water into urea or guanidine hydrochloride solutions is calculated from the relationship

$$\Delta G_{tr} = -RT \ln (S/S_{H_2O}) = -RT \ln (D/D_{H_2O}) \quad (1)$$

where S and S_{H_2O} refer to solubility in denaturant solution and water, respectively. Similarly, D and D_{H_2O} are the distribution coefficients in denaturant solution and water, respectively. The ΔG_{tr} values in urea solutions at 25 °C for the four peptides and ethyl acetate are shown in Figure 3. The free energies of transfer of these compounds become more negative with increasing urea concentration, and there is a linear relationship between the free energy of transfer and the number of peptide groups at all urea concentrations. Therefore, within the series of compounds the Δg_{tr} value of the peptide group for the transfer from water to urea is negative and is an *additive* function of the number of peptide groups present [e.g., −130 cal mol^{-1} (peptide group)$^{-1}$ in 8 M urea at 25 °C compared to the values of −60 and −310 cal/mol obtained from diglycine–glycine and triglycine–diglycine, respectively, by Nozaki & Tanford (1963); the contribution of each peptide group to the ΔG_{tr} has been otained from the slopes of the plots in Figure 3 in our study]. A similar linear relationship between

[4] The values of the ΔG_{tr} of the peptide derivatives from water to denaturant solutions at three different temperatures are available on request.

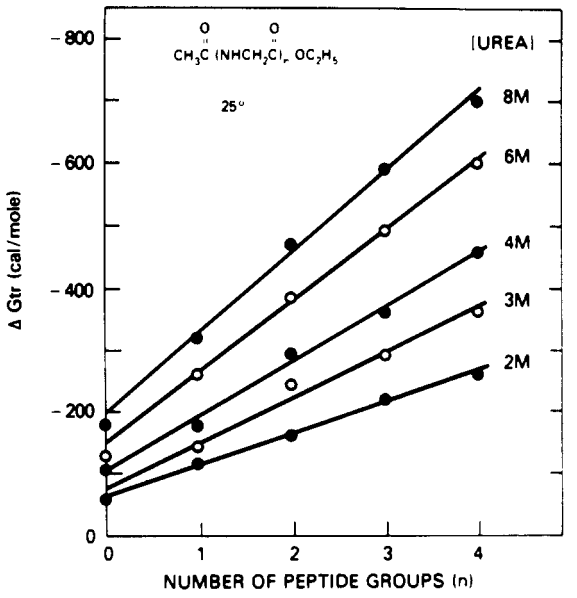

FIGURE 3: Dependence of free energy transfer, ΔG_{tr}, on the number of peptide groups in the acetylglycine derivatives at various urea concentrations. The values of the free energy of transfer per peptide group can be obtained from the slopes of the plots.

Table II: Contribution of a Peptide Residue (Δg_{tr}) in cal/mol to Free Energy of Transfer from Water to Aqueous Urea Solution at Three Temperatures[a]

urea (M)	from ΔG_{tr} values of Ag_1E, EtAc	Ag_2E, Ag_1E	Ag_3E, Ag_2E	Ag_4E, Ag_3E	av
		0 °C			
2		−90[b]	−85[b]	−85	−85
3		−105	−135	−105[c]	−115
4		−105	−165	−105	−125
6		−170	−190	−150	−170
		25 °C			
2	−60	−45	−55	−40	−50
3	−65[d]	−100	−45	−70	−70
4	−70	−115	−65	−95	−85
6	−135	−120	−105	−110	−115
8	−140	−150	−120	−110	−130
		40 °C			
2		−55	−35	−70	−55
3		−65	−55	−60[c]	−60
4		−75	−75	−80	−80
6		−120	−80	−80	−100

[a] The values of ΔG_{tr} were obtained as mentioned in the text. [b] From the extrapolated values of Ag_2E. [c] From the extrapolated values of Ag_4E. [d] From the extrapolated values of EtAc.

the number of peptide groups and the ΔG_{tr} values for the four acetylglycine esters obtained at 25 °C is also observed at 0 and 40 °C (data at these temperatures are not available for ethyl acetate). We have also obtained the Δg_{tr} per peptide group at 0 and 40 °C from similar plots as in Figure 3 (not shown). The Δg_{tr} per peptide group increases with decreasing temperature at all urea concentrations, indicating an exothermic nature of the interaction between urea and the peptides (Table II). The extent of exothermic interaction, however, decreases with an increase in temperature.

In contrast to the results in urea, plots of ΔG_{tr} as a function of the number of peptide groups in Gdn·HCl are nonlinear and the slopes increase with an increase in the number of the peptide group in the molecule (Figure 4). The contribution of each peptide groups to the negative free energies of the compounds in guanidine hydrochloride solutions is *not additive* but increases with the increase in the number of peptide groups

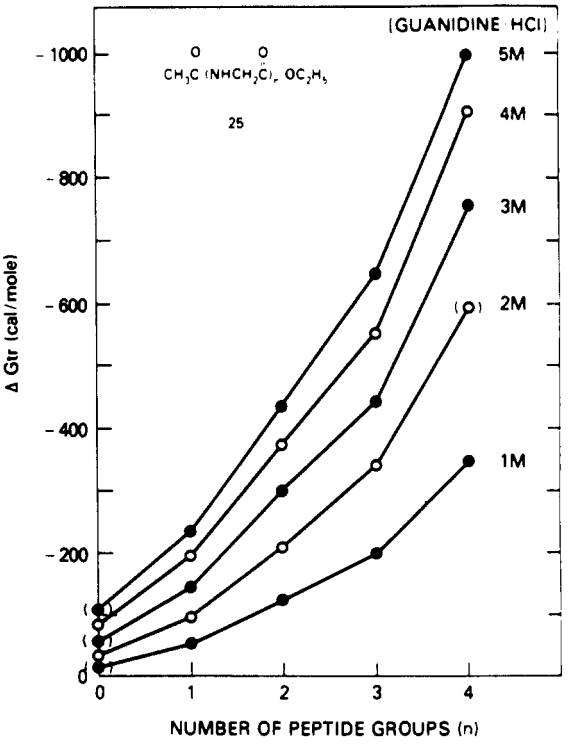

FIGURE 4: Variation of the free energy of transfer, ΔG_{tr}, on the number of peptide groups in acetylglycine derivatives at various guanidine hydrochloride concentrations.

Table III: Contribution of a Peptide Residue (Δg_{tr}) in cal/mol to Free Energy of Transfer from Water to Guanidine Hydrochloride Solution

Gdn·HCl (M)	from ΔG_{tr} values of Ag_1E, EtAc	Ag_2E, Ag_1E	Ag_3E, Ag_2E	Ag_4E, Ag_3E
		0 °C		
1		−100	−215	
2		−170	−345	
3		−250	−370	
4		−270	−405	
		25 °C		
1	−30[a]	−65	−80	−145
2	−60	−115	−130	−250[b]
3	−75[a]	−145	−150	−315
4	−110	−165	−190	−350
5	−120[a]	−205	−205	−350
		40 °C		
1		−85	−65	
2		−130	−90	
3		−150	−165	
4		−190	−210	

[a] From the extrapolated values of EtAc. [b] From the extrapolated value of Ag_4E.

in these compounds. The Δg_{tr} values increased from −110 cal mol^{-1} $peptide^{-1}$ (Ag_1E–EtAc) to −350 cal mol^{-1} $peptide^{-1}$ (Ag_4E–Ag_3E) in 5 M Gdn·HCl. The Δg_{tr} values for a peptide group in 4 M Gdn·HCl at 25 °C are −165 and −190 cal/mol from Ag_2E–Ag_1E and Ag_3E–Ag_2E, respectively, compared to −175 and −395 cal/mol obtained from diglycine–glycine and triglycine–diglycine combinations (Nozaki & Tanford, 1970). Similar nonlinear plots, the slopes of which increase with the increase in the number of peptide groups, are also observed at 0 and 40 °C (not shown; see, however, Table III). There is also a tendency for the ΔG_{tr} values to become more negative with decreasing temperature in Gdn·HCl as in urea solutions, but the temperature effects were generally negligible for all compounds between 25 and 40 °C and for Ag_1E at all tem-

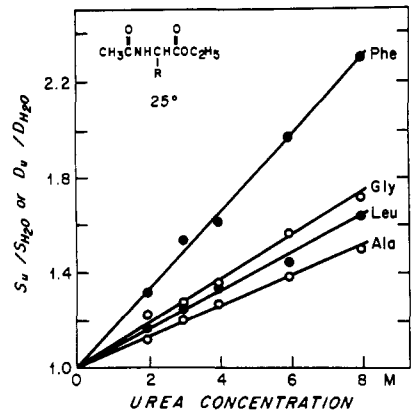

FIGURE 5: Effect of urea on the solubility increases of acetylglycine ethyl esters with side chains. For comparison, the solubility values of Ag_1E also have been included. Ala, AAE; Leu, ALE; Phe, APE.

peratures. Significant differences were seen between 0 and 25 °C for Ag_2E, and these became larger with Ag_3E over this temperature range.

The conclusion that can be drawn from the above results obtained from uncharged model compounds is that the additivity is observed for the transfer of the peptide group from water to urea solutions but not to Gdn·HCl solutions. Nozaki & Tanford (1963, 1970) from their amino acid studies did not observe additivity of Δg_{tr} for the transfer of the peptide group in both urea and Gdn·HCl solutions. Lapanje et al. (1978), however, have observed the additivity of Δg_{tr} for the peptide group when they calculated the values from triglycine–diglycine and tetraglycine–triglycine combinations. The values of the free energy of a peptide group obtained in our study are less than those obtained from charged amino acids in both denaturant solutions.

Effects on Hydrocarbon Side Chains. As in the series Ag_nE, an increase in urea concentration increased the solubility of acetylalanine, -valine, -norvaline, -leucine, -norleucine, and -phenylalanine ethyl esters. In Figure 5 a few of the results for these compounds at 25 °C have been shown together with the glycine derivative. It can be seen that up to the leucine ethyl ester, the increase in solubility is linear but is less than the solubility increase of Ag_1E up to 8 M urea. Only APE shows increased solubility greater than that of Ag_1E at all urea concentrations. Similar linear relationship in the increase in the solubility of the compounds is observed in urea solutions at 0 and 40 °C. However, the effectiveness of urea in increasing the solubility of these derivatives with a side chain increases with temperature in the range 0–40 °C (not shown), indicating a nonpolar hydrophobic interaction effect (Kauzmann, 1959; Roseman & Jencks, 1975). The values of the Δg_{tr} for various side chains obtained by subtracting the free energy value of Ag_1E from the transfer values of the side chain derivatives at three temperatures are shown in Table IV. The values for Ala, Val, and Nva side chains are positive in all urea concentrations at the temperatures studied here (for Val only 25 °C data are available). There is no definite trend in the free energy values with urea concentrations, although there is a tendency of Δg_{tr} becoming less unfavorable as the length of the chain increases. The Δg_{tr} values for the Leu side chain are positive at 25 °C, but the transfer becomes favorable at 40 °C. Norleucine, which has an unfavorable value at 0 °C, shows increased favorable transfer at higher temperatures. The Δg_{tr} values for the phenylalanyl group is negative at all urea concentrations between 0 and 40 °C and show a definite trend of increasing favorable interaction with an increase in temperature (endothermic).

Table IV: Contribution of Side Chains (Δg_{tr}) in cal/mol to Free Energy of Transfer from Water to Urea Solution at Three Temperatures

urea (M)	from ΔG_{tr} values of: AAE, Ag_1E, Ala[a]	AVE, Ag_1E, Val[a]	AnVE, Ag_1E, Nva[a]	ALE, Ag_1E, Leu[a]	AnLE, Ag_1E, Nle[a]	APE, Ag_1E, Phe[a]
			0 °C			
2	70		60		35	−35
3	65		55		40	−80
4	85		115[a]		85	−65
6	110		135		90	−90
			25 °C			
2	55	50	40	25	5	−45
3	35	25	25	15	−15	−110
4	40	35	25[b]	5	−25	−105
6	65	45	65	45	−10	−140
8	80	45	50	25	−20	−175
			40 °C			
2	25		20	−40	−45	−120
3	55		15	−20	−55	−145
4	80[b]		5[b]	−25[b]	−60[b]	−190
6	80		−10	−40	−90	−245

[a] Side chain. [b] Obtained from intrapolation.

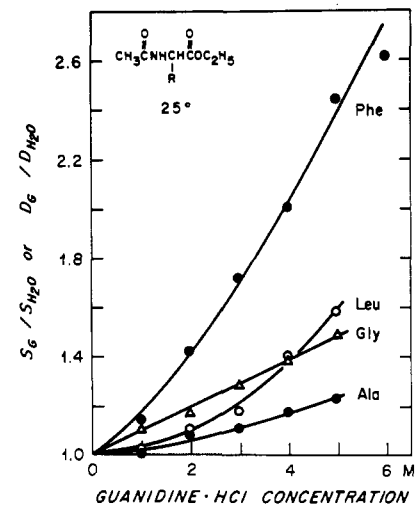

FIGURE 6: Effect of guanidine hydrochloride concentrations on the solubility increase of acetylglycine ethyl esters with side chains. For comparison, the solubility values of Ag_1E also have been included.

In Gdn·HCl, all these side chain compounds show nonlinear increases in solubility with increasing reagent concentration, the results for a few of which are presented in Figure 6. AAE, AVE, AnVE, ALE, and AnLE in 4 M Gdn·HCl show an increase in solubility that is less than the solubility increase of Ag_1E. APE showed considerably increased solubility in Gdn·HCl solution, and at higher concentrations Gdn·HCl is more effective than urea. The nonlinear increase in the solubility of the side chain esters in Gdn·HCl may arise from the salting out effect of the counter ion Cl^- (Nandi & Robinson, 1972b). Wetlaufer et al. (1964) also observed a salting out effect in the solubility of butane at lower Gdn·HCl concentrations. The effect of Gdn·HCl at 0 and 40 °C is similar to the results of 25 °C (not shown), and as in urea solution the solubility ratio increases with an increase in the temperature. In general, at higher Gdn·HCl concentration the transfer of the straight chain derivatives, viz., Nle and Nva, is energetically more favorable in denaturant solution than their corresponding branch chain derivatives. The Δg_{tr} values for various side chains are shown in Table V. As in urea solutions, most of the transfer values are positive for the aliphatic side

Table V: Contribution of Side Chains (Δg_{tr}) in cal/mol to Free Energy of Transfer from Water to Guanidine Hydrochloride Solution at Three Temperatures[a]

Gdn·HCl (M)	from ΔG_{tr} values of AAE, Ag_1E, Ala[a]	AVE, Ag_1E, Val[a]	AnVE, Ag_1E, Nva[a]	ALE, Ag_1E, Leu	AnLE, Ag_1E, Nle[a]	APE, Ag_1E, Phe[a]
			0 °C			
1	60				40	−25
2	110		110		105	−75
3	135		130		85	−145
4	195		150		100	
			25 °C			
1	55	60	50	45	45	−45
2	50	60	40	40	35	−115
3	85	65	85	45	10	−175
4	95	70	30	−5	−20	−220
5	115	65	−10	−35	−75	−295
			40 °C			
1	50		40	20	10	−40
2	95		35	25	−30	−130
3	80		15	10	−80	−185
4	80		−20	−65	−165	−260

[a] Side chain.

Table VI: Free Energies of Transfer (Δg_{tr}) of Nonpolar Side Chains into 8 M Urea and 5 M Guanidine Hydrochloride at 25 °C

solvent	side chain	amino acids[a]	hydrocarbons[b]	acetyl amino acid ethyl esters
urea	Phe	−600	−630[c]	−175[d]
	Leu	−295		25
	Val	−310	−350	45
Gdn·HCl	Phe	−640	−665	−295
	Leu	−375	−370	−35

[a] Nozaki & Tanford (1963). [b] Wetlaufer et al. (1964). [c] Phenyl group. [d] Herskovits et al. (1970) obtained values of 1.27 and 1.92 for the ratio of the solubility of *N*-acetyltryptophan ethyl ester at 25 °C in 1 and 3 M urea solutions, which correspond to a free energy of transfer to −127 and −385 cal/mol, respectively. Subtracting the transfer value of Ag_1E in 1 and 3 M urea, we obtain values of −50 and −240 cal/mol, respectively, for the tryptophan group. The corresponding intrapolated values for the tryptophan group are −160 and −380 cal/mol obtained by Nozaki & Tanford (1963).

chains. Only Leu and Nle side chains show favorable transfer at higher denaturant concentrations and at higher temperatures. The phenylalanine side chain shows favorable Δg_{tr} values at all temperatures and is endothermic as is observed in urea. The Δg_{tr} values are more favorable in Gdn·HCl than in urea, e.g., −220 and −105 cal/mol, respectively, at 25 °C for the phenylalanine group in 4 M denaturant solutions.

In Table VI, we have presented the Δg_{tr} values of a few of the side chain groups for the transfer to the denaturant solution from the literature and compared them with the results of the present study. Not only do we not observe the additivity of the side chains for the transfer to urea solutions, but also the Δg_{tr} values up to 8 M urea concentrations for the aliphatic side chains are *positive*, making them unfavorable for the transfer to urea solutions. The aromatic phenylalanine group (which was only studied here) in urea solutions and the large aliphatic side chains at higher Gdn·HCl concentrations show favorable free energy of transfer. However, serious lack of additivity is still observed. Lapanje et al. (1978) also observed the lack of additivity of the Leu side chain obtained from various combinations of oligoleucines. From our results with model peptide esters, it seems that the contributions of the nonpolar side chain to free energies of model compounds are much smaller than previously estimated by other investigators from measurements based on amino acids and hydrocarbons

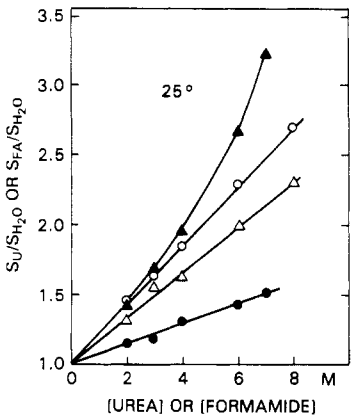

FIGURE 7: Comparison of the effects of formamide and urea on the solubility increase of acetyltriglycine and acetylphenylalanine ethyl esters at 25 °C. S_{FA}, solubility in formamide. Ag_3E: (○) urea; (●) formamide. APE: (△) urea; (▲) formamide.

(see also footnote *d* of Table VI).

A general conclusion that can be made from the present study is that because of the specific structural effects and the lack of additivity, it will be difficult or impossible to make quantitative estimates of what has to be expected for free energies of protein denaturation. Perhaps such estimates can be made if a sufficient number of free energies are obtained for the different individual amino acids, especially if the sequence of the protein is known and can be correlated with measured effects observed with suitable peptides.

Effects of Formamide. The effect of formamide on the solubility of two representative amino acid derivatives, e.g., Ag_3E and APE, was studied. It can be seen from Figure 7 that formamide is less effective than urea in increasing the solubility of Ag_3E. Robinson & Jencks (1965a) also observed that formamide was less effective in increasing the solubility of Ag_4E compared to urea. In contrast, formamide is much more effective than urea in increasing the solubility of acetylglycine ester with a nonpolar moiety, i.e., APE, than urea (Figure 7). Roseman & Jencks (1975) also found that although formamide was less effective than urea in increasing the solubility of the polar uric acid molecule, the nonpolar molecule naphthalene showed increased solubility in formamide compared to urea.

Mechanism of Interaction. The mechanism of protein denaturation induced by urea and Gdn·HCl has been the subject of a large number of investigations (Tanford, 1968, 1970; Pace, 1975; Eagland, 1975; Franks & Eagland, 1975; Prakash et al., 1981; Herskovits et al., 1983). The mechanism has developed mainly based on (i) the modification of water structure by the denaturant molecule and (ii) the stoichiometric interaction of the denaturant molecules with the peptide groups in proteins or both of these effects. The explanation of the mechanism was obtained mostly from the study of the model compounds in these solutions. As has already been mentioned, both urea and Gdn·HCl increase the solubility of hydrocarbons and amino acid side chains. In addition, these denaturant molecules can also have favorable interaction with polar peptide groups in water. The favorable interaction of these groups with urea or Gdn·HCl solutions may represent a decrease in the activity coefficient or stoichiometric complex formation between the solute and the denaturant molecules or both (Roseman & Jencks, 1975). Based on a variety of experimental approaches, it has been considered that modification of water structure by urea (for which most of the data are available) would make the insertion of the nonpolar groups easier in denaturant solution than in water [Frank & Franks,

1968; Herskovits (1973), and more than 20 references in Roseman & Jencks (1975)] and the binding of urea or Gdn·HCl with the peptide groups or combination of both these factors can explain the denaturation of protein molecules.

The effect of urea on the breaking of solvent water structure and its effect on the solubility of the nonpolar molecules and groups have come under increased scrutiny in recent years (Rosemann & Jencks, 1975). In addition, precise information on the equilibrium structure of water around nonpolar groups in proteins is not available (von Hippel & Hamabata, 1973).

There is some evidence that urea can form a complex with the peptide group. Swenson & Koob (1963) from the NMR study of urea with peptides and Uedaira (1972) from the X-ray refraction intensity curves considered the presence of a complex between urea and peptide groups. The circular dichroic spectra of polypeptides and protein in urea and Gdn·HCl solutions have been interpreted as arising from binding of these molecules to an extended helix of the polypeptide chain (Tiffany & Krimm, 1973). Schonert & Stroth (1981) from the IR spectra of the amide I band in the presence of urea and from δ H and δ C shifts of the peptide groups in urea could not reach any definite conclusion regarding complexation, although they concluded complex formation between urea and peptide group from calorimetric studies.

Robinson & Jencks (1965a) from the solubility studies of Ag_4E in urea and Gdn·HCl suggested complex formation between the peptide, $-CH_2CO-NH-$, and the denaturant molecules by bifunctional H bonding as monofunctional H bonding does not have stability in water (Klotz & Franzen, 1964; Gill & Noll, 1972). Nozaki & Tanford (1970) considered the possibility of bifunctional H bonding to explain the results of solubility of amino acids in urea and Gdn·HCl solutions. Roseman & Jencks (1975) could not explain the increased solubility of uric acid in urea and Gdn-HCl by bifunctional H bonding, since the geometry of the uric acid molecule would prevent the formation of such bonds. These authors, from extensive study of the solubility behavior of uric acid and a nonpolar solute naphthalene in polar and relatively nonpolar solvents, suggested that the driving force for the favourable interaction effect of cosolvent with both polar and nonpolar solutes arises from a more favorable sum of free energies of cavity formation and nonpolar interaction (ΔG^{cav} + $\Delta G^{int}{}_{nonpolar}$) in the presence of cosolvent. With polar H-bonding solutes, the cosolvents must also be able to interact through H bonding ($\Delta G^{int}{}_{polar}$), which would prevent a net loss of H-bond interaction. Roseman & Jencks (1975) formulated that the free energy of transfer of a solute from water to a water–cosolvent mixture is given by

$$\Delta G_{tr} = (\delta\Delta G^{cav} + \delta\Delta G^{int}{}_{nonpolar}) + \delta\Delta G^{int}{}_{polar} \qquad (2)$$

in which $\delta\Delta G$ term represents the difference in the free energy of that compound in two solvents.

The increased solubility of the acetylglycine derivatives (with and without side chains) in urea may arise from the first term ($\delta\Delta G^{cav} + \delta\Delta G^{int}{}_{nonpolar}$), which is favorable for all solutes, polar or nonpolar, in denaturant solutions. The exothermic nature of transfer of the derivatives (without side chains) to urea and Gdn·HCl solutions would suggest an exchange of a peptide–water H bond for a peptide–amide H bond. Roseman & Jencks (1975) also suggested that mono- as well as bifunctional hydrogen bonding was possible between the solutes and the denaturant molecules (included in the $\delta\Delta G^{int}{}_{polar}$ term). The relatively larger increase in the solubility of Ag_3E in urea than formamide observed here and the similar observation for AG_4E by Robinson & Jencks (1965a) may suggest the formation of a bifunctional hydrogen bond between urea and these peptides.

For the side chain derivatives from Ala to Leu, the first term of eq 2, as with the unsubstituted derivatives, is energetically favorable; but due to the presence of nonpolar moiety attached to the peptide group, the extent of hydrogen-bond formation, which is possible for the unsubstituted derivatives, may not be possible with the side chain derivatives. This would explain the relatively less ΔG_{tr} for the transfer of the side chain compounds than Ag_1E. Urea has been found to decrease the solubility of small-sized methane although the solubility of propane and butane is increased in urea solution compared to that in water (Wetlaufer et al., 1964). This would mean that with larger groups there is a favorable effect and may explain the less unfavorable ΔG_{tr} for the valine and leucine than the alanine derivative. In the presence of the aromatic phenyl group in APE, the ΔG_{tr} term becomes favorable (due to more favorable $\delta\Delta G^{int}$ nonpolar term for the unsaturated moiety) due to dispersion and other interactions (Roseman & Jencks, 1975). This may also reflect the fact that the benzene ring has a considerable surface area but does not take up a very large volume in the solvent. Formamide, which can fit in the water structure more efficiently than urea, probably offers a more favorable surface of interaction to the aromatic groups than urea (Frank & Franks, 1968).

The effect of Gdn·HCl toward the increased solubility of the compounds can be explained following the same consideration as has been suggested for urea. The increased effectiveness for the Ag_nE derivatives may have resulted from the greater effectiveness of Gdn·HCl in forming bifunctional H bonds with the peptide groups (Robinson & Jencks, 1965a). In addition to the effects described for urea solution, the salting out of the nonpolar group by Cl^- in Gdn·HCl may also contribute to the less favorable ΔG_{tr} for side chain derivatives and can also explain the nonlinear increase in solubility at lower Gdn·HCl concentrations (see above). Wetlaufer et al. (1964) have observed a similar salting out contribution in the solubility of butane in Gdn·HCl due to the Cl^- ion.

Registry No. Ag_1E, 1906-82-7; Ag_2E, 3757-98-0; Ag_3E, 35433-67-1; AAE, 5686-40-8; AnVE, 35433-65-9; AVE, 2382-78-7; AnLE, 35433-66-0; ALE, 1114-55-2; APE, 2361-96-8; urea, 57-13-6; guanidine hydrochloride, 50-01-1; formamide, 75-12-7.

References

Almog, R., Schrier, M. Y., & Schrier, E. E. (1978) *J. Phys. Chem. 82*, 1703–1707.

Cesaro, A., Russo, E., & Barone, G. (1982) *Int. J. Pept. Protein Res. 20*, 8–15.

Eagland, D. (1975) in *Water* (Franks, F., Ed.) pp 305–518, Plenum Press, New York.

Eagland, D., & Pilling, J. (1980) *Biopolymers 19*, 147–164.

Formisano, S., Johnson, M. L., & Edelhoch, H. (1978) *Biochemistry 17*, 1468–1473.

Frank, H. S., & Franks, F. (1968) *J. Chem. Phys. 48*, 4746–4757.

Franks, F., & Eagland, D. (1975) *CRC Crit. Rev. Biochem. 3*, 165–219.

Gill, S. J., & Noll, L. (1972) *J. Phys. Chem. 76*, 3065–3068.

Herskovits, T. T. (1973) *J. Phys. Chem. 77*, 381–386.

Herskovits, T. T., Jaillet, H., & Gadegbeku, B. (1970) *J. Biol. Chem. 245*, 4544–4550.

Herskovits, T. T., Geotge, R. C. S., & Erhunmwunsee, L. J. (1981) *Biochemistry 20*, 2580–2587.

Herskovits, T. T., Jacobs, R., & Nag, K. (1983) *Biochim. Biophys. Acta 742*, 142–154.

Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1–57.

Klotz, I. M., & Franzen, J. S. (1962) *J. Am. Chem. Soc. 84*, 3461–3466.

Lapanje, S., Skerjanc, J., Glavnik, S., & Zibert, S. (1978) *J. Chem. Thermodyn. 10*, 425-433.
Nandi, P. K. (1974) *J. Phys. Chem. 78*, 1197-1203.
Nandi, P. K. (1976) *Int. J. Pept. Protein Res. 8*, 253-264.
Nandi, P. K., & Robinson, D. R. (1972a) *J. Am. Chem. Soc. 94*, 1299-1308.
Nandi, P. K., & Robinson, D. R. (1972b) *J. Am. Chem. Soc. 94*, 1308-1315.
Nemathy, G. (1967) *Angew. Chem., Int. Ed. Engl. 6*, 195-206.
Nozaki, Y., & Tanford, C. (1963) *J. Biol. Chem . 238*, 4074-4081.
Nozaki, Y., & Tanford, C. (1970) *J. Biol. Chem. 245*, 1648-1652.
Pillon, W. N., & Bertles, J. F. (1979) *J. Biol. Chem. 254*, 3462-3467.
Prakash, V., Loucheux, C., Scheufele, S., Gorbunoff, M., & Timasheff, S. N. (1981) *Arch. Biochem. Biophys. 210*, 455-464.
Robinson, D. R., & Jencks, W. P. (1965a) *J. Am. Chem. Soc. 87*, 2462-2470.
Robinson, D. R., & Jencks, W. P. (1965b) *J. Am. Chem. Soc. 87*, 2470-2479.
Roseman, M., & Jencks, W. P. (1975) *J. Am. Chem. Soc. 97*, 631-640.
Schonert, H., & Stroth, L. (1981) *Biopolymers 20*, 817-813.
Schrier, M. Y., & Schrier, E. E. (1976) *Biochemistry 15*, 2607-2612.
Swenson, C. A., & Koob, L. (1970) *J. Phys. Chem. 74*, 3376-3380.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 122-181.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1-95.
Tiffany, M. L., & Krimm, S. (1973) *Biopolymers 12*, 575-587.
Uedaira, H. (1972) *Bull. Chem. Soc. Jpn. 45*, 3068-3072.
von Hippel, P. H., & Hamabata, A. (1973) *J. Mechanochem. Cell Motil. 2*, 127-138.
Wetlaufer, D. B., Malik, S., Stoller, L., & Coffin, R. L. (1964) *J. Am. Chem. Soc. 86*, 508-514.
Wolf, J. P., III, & Nieman, C. (1963) *Biochemistry 2*, 493-497.

Dimer Structure of the Ribosomal Protein L7/L12 Probed by Energy Transfer†

Ton P. G. M. Thielen,* J. Antonie Maassen, Jan Kriek, and Wim Möller

ABSTRACT: In dimers of the ribosomal protein L7/L12 from *Escherichia coli*, we have measured the transfer of excitation energy between fluorescent probes and derived therefrom the distances between the labeling sites. *N*-[7-(Dimethylamino)-4-methylcoumarinyl]maleimide (DACM) served as the energy donor to fluorescein. The probes were coupled covalently and with great specificity either to the N-terminal serine or to Lys-51. In one set of experiments, DACM and fluorescein were located on different chains of the L7/L12 dimer. The distance between the two labels was 45 ± 5 Å at the position of the Lys-51 residues and 33 ± 5 Å at the N-termini. These long distances provide additional evidence for a staggered conformation of the L7/L12 dimer. Comparison of the distances determined in free L7/L12 with those determined in L7/L12 bound to 50S ribosomes showed that no major changes occur in the probed region of L7/L12 upon binding to the ribosome. We also measured energy transfer in L12 labeled both with DACM, at position 51, and with fluorescein, at the N-terminus. In dilute solution, this protein was monomeric; the distance between the labels was at least 75 Å. On 50S ribosomes, a distance of about 45 Å was measured, but since almost 2 equiv of the labeled protein was bound, most likely the distance between DACM and fluorescein on different polypeptides was found. A model of the dimer structure of L7/L12 is presented, which is consistent with all experimental data available.

Like their counterparts from eukaryotic and other prokaryotic ribosomes, the acidic proteins L7 and L12 from *Escherichia coli* are present in multiple copies per ribosome (Kurland, 1977; Matheson et al., 1980; Liljas, 1982). Localized in a stalklike projection of the ribosome (Lake, 1976; Strycharz et al., 1978; Möller et al., 1983), they appear to form a flexible region (Tritton, 1978; Gudkov et al., 1982). They modulate the interaction of the ribosome with several of the initiation, elongation, and termination factors, and as such, they are indispensable for speed and fidelity of protein synthesis (Möller, 1974; Pettersson & Kurland, 1980). Except for the N-terminal acetyl group of L7, the two proteins are the same (Terhorst et al., 1972). Since L7 and L12 are also identical functionally (Möller, 1974), they are normally referred to as L7/L12. In solution, L7/L12 forms dimers (Möller et al., 1972; Wong & Paradies, 1974; Gudkov et al., 1977). On the ribosome, the four copies of L7/L12 (Subramanian, 1975; Pettersson et al., 1976) are bound in the form of two dimers (Koteliansky et al., 1978; Zantema et al., 1982a).

Because of its remarkable features, L7/L12 has attracted much attention. Spectroscopic and hydrodynamic studies on the free dimers indicated an elongated structure (Wong & Paradies, 1974; Österberg et al., 1976) with a high content of α-helix (Dzionara, 1970; Möller et al., 1970; Gudkov et al., 1978; Luer & Wong, 1979). The formation of dimers was found to be due to aggregation of the N-terminal half of the molecule (Van Agthoven et al., 1975; Gudkov & Behlke, 1978; Koteliansky et al., 1978). The three-dimensional structure of the crystallizable C-terminal half has been elucidated (Leijonmarck et al., 1980). Although in most of the resulting

† From the Department of Medical Biochemistry, Sylvius Laboratories, 2333 AL Leiden, The Netherlands. *Received May 7, 1984.* This investigation was supported by the Organization for the Advancement of Pure Research (ZWO) and the Netherlands Foundation for Chemical Research (SON).

0006-2960/84/0423-6668$01.50/0