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Direct measurement of salt-bridge solvation energies using a peptide model system: Implications for protein stability

(protein folding/electrostatics/hydrophobicity/octanol partitioning/^{13}C NMR)

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ABSTRACT **The solvation energies of salt bridges formed between the terminal carboxyl of the host pentapeptide AcWL-X-LL and the side chains of Arg or Lys in the guest (X) position have been measured. The energies were derived from octanol-to-buffer transfer free energies determined between pH 1 and pH 9. ^{13}C NMR measurements show that the salt bridges form in the octanol phase, but not in the buffer phase, when the side chains and the terminal carboxyl group are charged. The free energy of salt-bridge formation in octanol is approximately −4 kcal/mol (1 cal = 4.184 J), which is equal to or slightly larger than the sum of the solvation energies of noninteracting pairs of charged side chains. This is about one-half the free energy that would result from replacing a charge pair in octanol with a pair of hydrophobic residues of moderate size. Therefore, salt bridging in octanol can change the favorable aqueous solvation energy of a pair of oppositely charged residues to neutral or slightly unfavorable but cannot provide the same free energy decrease as hydrophobic residues. This is consistent with recent computational and experimental studies of protein stability.**

The contribution of salt bridges to protein stability depends on their location (1–3). Salt bridges exposed to solvent generally have a marginal effect on stability (4–6), whereas buried salt bridges can provide significant stabilization (7, 8) or destabilization (9) relative to polar but uncharged residues. Hendsch and Tidor (10), in a computational study of electrostatic free energies, found that most of the 21 partially buried salt bridges they examined should be destabilizing, relative to hydrophobes of the same size because the high energy cost of desolvation cannot be completely canceled by salt-bridge and other interactions within the protein. They thus proposed that the replacement of buried salt bridges by hydrophobic residues should generally increase protein stability. Their proposal was subsequently confirmed by Waldburger *et al.* (8) who found that the stability of Arc repressor protein increased when a fully buried stabilizing salt-bridged triad was replaced by a hydrophobic triad. Although these complementary studies generally validate the computational approach, direct experimental measurements of both solvation and salt-bridge energetic in model peptide systems are a necessary step for refining our understanding of the thermodynamics of salt bridges. We present here the results of such measurements.

In the course of studying the solvation energies of guest (X) amino acids in the host pentapeptide AcWL-X-LL, we found (11) that the partitioning of the Arg and Lys peptides between octanol and water was anomalous: They were about as hydrophobic as the Trp peptide at high pH values and their transfer free energies were essentially unchanged over the pH range where we expected large changes to result from protonation of the carboxyl group. We report here ^{13}C NMR studies that demonstrate that the anomalous behavior is due to the formation in the octanol phase of an intramolecular salt bridge between the Arg and Lys side chains and the terminal carboxyl group. This model system thus permits the solvation and salt-bridge energies to be evaluated separately. The results show that the free energy of salt-bridge formation is about −4 kcal/mol (1 cal = 4.184 J), which approximately equals the solvation energy cost of two oppositely charged residues, taken relative to Gly. However, this salt-bridge energy is considerably smaller than that which would result from replacing the charged residues with Leu residues. These findings agree with experimental and computational studies of salt-bridge energetics in proteins that indicate that the contribution of buried salt bridges to stability is marginal compared to hydrophobic residues (8, 10).

MATERIALS AND METHODS

Peptide Synthesis and Partitioning. The host peptide AcWL-X-LL was chosen for studies (11) of the solvation energies of the 20 natural amino acids to satisfy our design criteria that all the partition coefficients be measurable and that they not be obscured by peptide aggregation or by changes in secondary structure. The experimental procedures for peptide synthesis and octanol–water partitioning have been described in detail (11). Briefly, the peptides were synthesized by using standard Fmoc methodology (12) and purified by using C_{18} reverse-phase HPLC. All peptides were >99% pure in both solvent systems and had the correct molecular weight by fast-atom-bombardment mass spectrometry. Volume-fraction octanol-to-water partition coefficients were measured by using Hepes buffer (10 mM Hepes/50 mM KCl/1 mM EDTA/3 mM NaN_3) at pH 9.0 and pH 1. Partition coefficients were measured 5–10 times for each peptide and were always found to be independent of peptide concentration, water-to-octanol volume ratio, and the area of the interface between the bulk phases. Volume fraction partition coefficients are defined by $K_v = P_b/P_o$, where P_b and P_o are the peptide concentrations in the buffer and octanol phases, respectively. Mole fraction partition coefficients (K_x) were calculated from the volume fraction (K_v) values by $K_x = K_v \cdot (v_{wat}/v_{oct})$, where v_{wat}/v_{oct} = 0.114 is the ratio of the molar volumes of water and octanol. All free energies are in mole fraction units and are given by $\Delta G = -RT \ln K_x$.

NMR Measurements. ^{13}C NMR spectra were recorded at 22°C on a Bruker DMX-500 spectrometer at 125.721 MHz. Typically, 16 scans were accumulated. Samples in water contained 10 vol % ^2H_2O for a deuterium lock signal. Chemical shifts of the peptides in water were calibrated relative to the ^{13}C signal of sodium 3-trimethylsilylpropionate. Spectra of the

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Abbreviation: ASA, accessible surface area.
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peptides in octanol were recorded without a deuterium lock. No field drift was noticed during the experiments. Chemical shifts were measured relative to the resonances of octanol. Chemical shifts of octanol signals were taken from Roberts *et al.* (13).

Monte Carlo Simulations. We examined the conformational space available to the AcWL-X-LL peptides (X = Lys$^+$, Arg$^+$, or His$^+$) by using Monte Carlo computer simulations with the Metropolis sampling algorithm (14). The methods employed have been described (11, 15). Conformations of the peptides were generated by making rotations about randomly selected torsions by random amounts (0 to $\pm 180°$) and displacing atoms by small random distances (≈ 0.005 Å). We used the AMBER/OPLS force field (16, 17) to describe the interactions within the peptide. This force field employs the united atom approximation; polar hydrogens are represented explicitly; nonpolar hydrogens are treated by increasing the radii of the heavy atoms to which they are bonded. The dielectric was set to that of bulk water ($\varepsilon = 78$) and the temperature was set to be 298 K. All of the simulations an equilibration period of 10^5 Monte Carlo iterations, with data being collected every 3000 steps from the subsequent 3×10^7 steps.

RESULTS AND DISCUSSION

Octanol-to-Water Free Energies of Transfer. The free energies of transfer of the AcWL-X-LL peptides with ionizable residues in the X position relative to the Gly peptide are summarized in Table 1. The peptides with basic residues are hydrophilic relative to Gly at pH 1 as expected, but surprisingly, the Arg and Lys peptides appear to be "hydrophobic" relative to Gly by 1.5–2 kcal/mol at pH 9. This observation was examined further by comparing the pH dependence of the partitioning of the Arg and Lys peptides with that of peptides with nonionizable residues in the X position. The comparison shown in Fig. 1, for example, reveals that partitioning of the Lys peptide is little affected by pH, whereas the partitioning of the Ser peptide is strongly affected because of protonation–deprotonation of the C terminus. The Ser-peptide data combined with similar data for the Gly, Ala, and Thr peptides yield an average value of 4.78 ± 0.06 (SEM) kcal/mol for the energetic cost of deprotonating the C terminus in the octanol phase (11). Why is the partitioning of the Lys peptide unaffected by pH? We hypothesized that salt-bridge formation was involved and considered two scenarios: (*i*) A strong Lys$^+$·COO$^-$ interaction in both phases prevents protonation–deprotonation of the carboxyl group. (*ii*) A strong Lys$^+$·COO$^-$ interaction in the octanol phase alone reduces the solvation energy of the deprotonated form to a value similar to that of the protonated form.

Table 1. Octanol-to-water transfer free energies of the host–guest peptides AcWL-X-LL containing ionizable residues in the X position relative to the AcWL-G-LL peptide

	pH 1.0		pH 9.0	
X residue	$\Delta\Delta G$, kcal/mol	ΔQ	$\Delta\Delta G$, kcal/mol	ΔQ
Asp	−0.26 ± 0.05	0	−3.47 ± 0.13	−1
Glu	−0.02 ± 0.03	0	−3.54 ± 0.15	−1
Arg	−1.86 ± 0.04	+1	+1.98 ± 0.02	+1
Lys	−2.82 ± 0.03	+1	+1.48 ± 0.02	+1
His	−2.32 ± 0.03	+1	−0.09 ± 0.02	0

Relative transfer free energies $\Delta\Delta G = \Delta G_{\mathrm{AcWLXLL}} - \Delta G_{\mathrm{AcWLGLL}}$ are derived from the absolute free energies obtained by Wimley *et al.* (11). ΔQ is the charge relative to the Gly peptide and thus represents the charge state of the X residue. The terminal carboxyl group and all ionizable side chains except His are charged at pH 9 while at pH 1 only Arg, Lys, and His are charged because all carboxyl groups are protonated. At pH 1, the peptides with basic residues are hydrophilic relative to Gly, as expected. Surprisingly, at pH 9, the Arg and Lys peptides appear to be "hydrophobic" relative to Gly by 1.5–2 kcal/mol. This effect is due to an intermolecular salt bridge that forms in the octanol phase between the Arg or Lys side chains and the C terminus (Fig. 2).

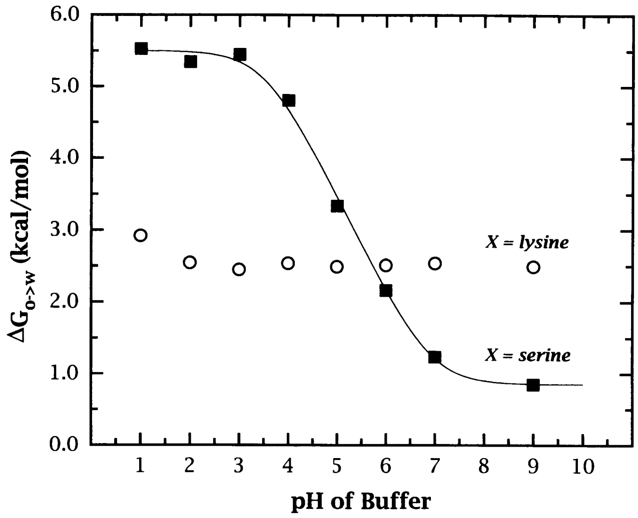

FIG. 1. pH dependence of the octanol-to-buffer transfer free energies $\Delta G_{O\to W}$ of AcWL-S-LL and AcWL-K-LL. The pH dependence of AcWL-S-LL partitioning indicates that the pK_a of the carboxyl group is 3.5 ± 0.1 and that the free energy difference between the charged and uncharged forms is 4.67 kcal/mol. $\Delta G_{O\to W}$ of AcWL-K-LL is essentially independent of pH, indicating that either the carboxyl group is not titrated over this pH range or that $\Delta G_{O\to W}$ of the protonated and deprotonated forms are very similar.

These explanations are possible only if it is conformationally feasible for the Lys or Arg side chains to come into close proximity with the terminal carboxyl group. We therefore performed Monte Carlo simulations using the OPLS force field to examine the conformational feasibility of Lys$^+$·COO$^-$, Arg$^+$·COO$^-$, or His$^+$·COO$^-$ interactions. The results indicated that such interactions were indeed possible as is shown by one of the conformations of the Lys peptide illustrated in Fig. 2. The Lys, Arg, and His side chains can easily approach to within 3–4 Å of the terminal carboxyl. Such distances are typical of salt bridges observed in proteins (18).

^{13}C NMR Measurements. We determined the pK_a of the ^{13}COOH group by using NMR chemical shift data obtained

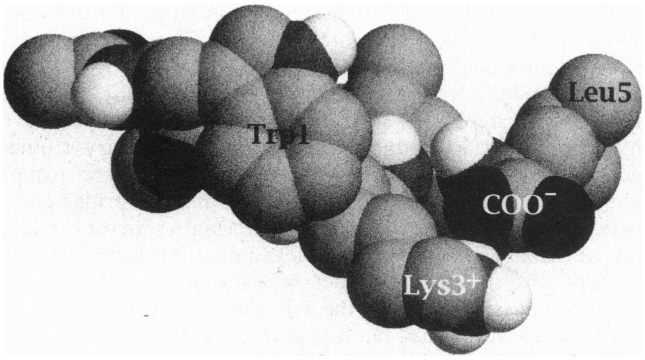

FIG. 2. Sample conformation of AcWL-K-LL obtained by using Monte Carlo simulations of the peptide *in vacuo*. The OPLS force field used in the simulation tends to give compact peptides because it accentuates electrostatic and van der Waals interactions. The primary purpose of the simulation was to examine the conformational feasibility of a salt bridge between Lys, Arg, or His and the terminal carboxyl group. The Lys in the conformation shown can approach the COO$^-$ to within 3.5–4.0 Å. Overall, the simulations show that it is feasible for Arg, Lys, and His to form a salt bridge with COO$^-$.

form the carboxyl-labeled Ser and Lys peptides in both the buffer and octanol phases. We equilibrated the octanol and buffer phases over a range of pH values and recorded ^{13}C spectra of the peptides in samples withdrawn from each phase at each pH. The behavior of AcWL-S-LL serves as a control because the Ser side chain is not ionizable. In all cases, the chemical shifts exhibit sigmoidal transitions as the carboxyl group is deprotonated as shown in Fig. 3. The transition curves of Fig. 3*A* immediately rule out the possibility of a $Lys^{+}\cdot COO^{-}$ salt bridge in the buffer phase because the Ser and Lys peptides have virtually identical pK_a values; any ionic interaction with the COO^{-} group would shift the pK_a of that group.

The pK_a of AcWL-S-LL in buffer is 3.73 ± 0.05 (Fig. 3*A*), while in octanol the apparent pK_a is increased to 6.96 ± 0.05 (Fig. 3*B*). These results and our free-energy-of-transfer data (11) permit a thermodynamic cycle for the pH dependence of the partitioning of AcWL-S-LL to be established as shown in Fig. 4*A*. Importantly, the difference of 4.67 kcal/mol between the ΔG values for the partitioning of the protonated and deprotonated forms of the peptide agrees well with the value of 4.4 kcal/mol calculated from $\Delta\Delta G = 2.303RT\Delta pK_a$.

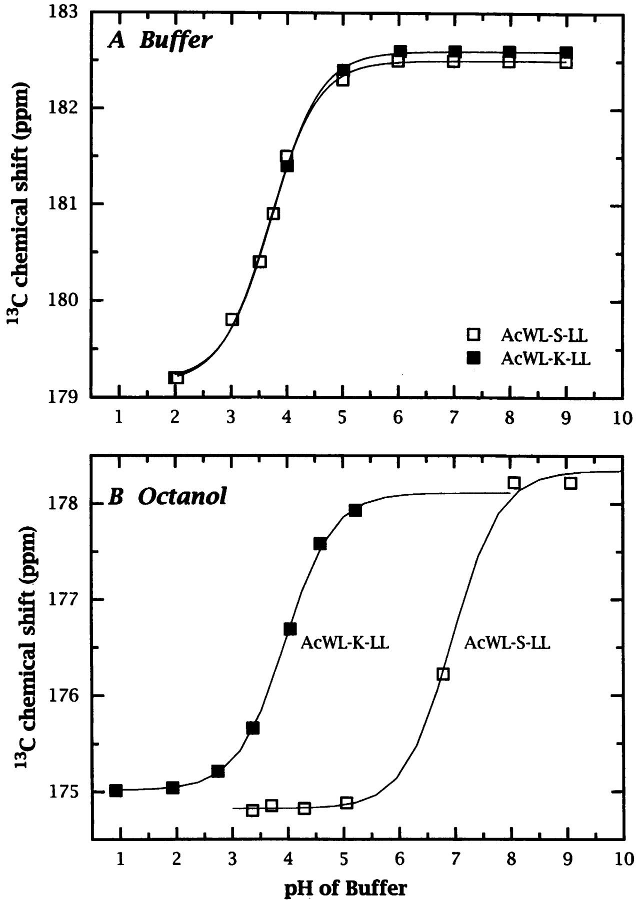

FIG. 3. pH dependence of the ^{13}C chemical shift of AcWL-S-LL and AcWL-K-LL labeled in the carboxyl group with ^{13}C. NMR spectra were collected from samples of buffer and octanol equilibrated at different pH values. At low pH values, the carboxyl groups of the peptides are protonated in both phases. At high pH values, the carboxyls are deprotonated in both phases. The pK_a values of the two peptides are identical (3.7 ± 0.1) in buffer, indicating that no interactions occur between the two ionized groups of AcWL-K-LL in buffer. In octanol, however, the pK_a of AcWL-K-LL is shifted down by ≈3 pH units relative to AcWL-S-LL. This means that there is an ionic interaction between the Lys side chain and the carboxyl group that lowers the free energy by 4.1 kcal/mol. A similar value is obtained from the thermodynamic analysis described in Fig. 5.

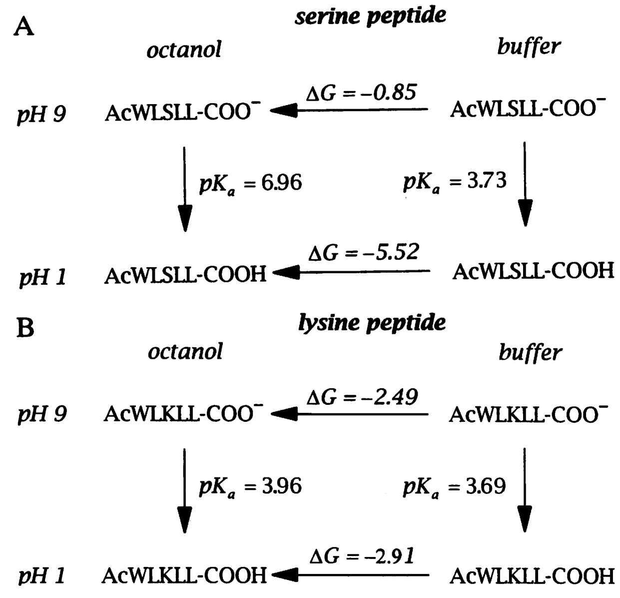

FIG. 4. Thermodynamic cycles depicting the water-to-octanol partitioning of AcWL-S-LL (*A*) and AcWL-K-LL (*B*) at high and low values of pH. The transfer free energies (11) of the protonated and deprotonated forms are indicated as horizontal steps and the pK_a values as vertical steps. The pK_a values in octanol are apparent values defined as the buffer pH at which the concentrations of protonated and deprotonated peptide are equal in octanol. In both cases the differences in ΔG are approximately equal to $-2.303RT\Delta pK_a$. Transfer free energies have units of kcal/mol.

For the AcWL-K-LL peptide, the sigmoidal change in the ^{13}C chemical shift (Fig. 3) demonstrates that in both phases the carboxyl group is protonated at low pH and deprotonated at high pH. The pK_a is 3.69 ± 0.05 in buffer while the apparent pK_a in octanol is 3.96 ± 0.05. These values indicate that the carboxyl group is fully protonated in both phases at pH 1, so that the relative transfer free energies for the Lys and Arg peptides at pH 1 shown in Table 1 measure the cost of transferring the Lys and Arg side chains from water to octanol. The thermodynamic cycle for the partitioning, summarized in Fig. 4*B*, shows that the small difference between the transfer free energies at pH 1 and pH 9 (0.43 kcal/mol) is reflected in the small difference in pK_a ($2.303RT\Delta pK_a$ = 0.34 kcal/mol).

The protonation state of the carboxyl group of AcWL-K-LL clearly changes as the pH is varied (Fig. 3) and yet the transfer free energies vary only slightly (Fig. 1). This is true of AcWL-R-LL as well (Table 1). Thus, there must be an energetic effect of Lys or Arg that compensates for the high free energy cost of transferring the deprotonated carboxyl group into octanol. The 3 pH unit change (ΔpK_a) in the apparent octanol pK_a of AcWL-K-LL relative to AcWL-S-LL indicates that the energetic effect is an electrostatic one because the free energy of the charged carboxyl in the octanol phase is decreased by $2.303RT\Delta pK_a$ = 4.1 kcal/mol. This electrostatic interaction is unlikely to be an intermolecular one because the transfer free energy of AcWL-K-LL is independent of peptide concentration between 2 μM and 100 μM (data not shown). Furthermore, it cannot be due to an interaction with anions in the buffer phase because the transfer free energy of AcWL-K-LL from distilled water (pH ≈6) is identical to that from the Hepes/KCl buffer. We therefore conclude that the terminal carboxyl group is stabilized in octanol through intramolecular salt-bridge interactions with the Arg or Lys side chains and presumably the His side chain as well.

Energetics of Salt-Bridge Formation. The free energy of the $Lys^{+}\cdot COO^{-}$ interaction that occurs in the octanol phase, −4.1 kcal/mol, is similar to the value obtained by means of the

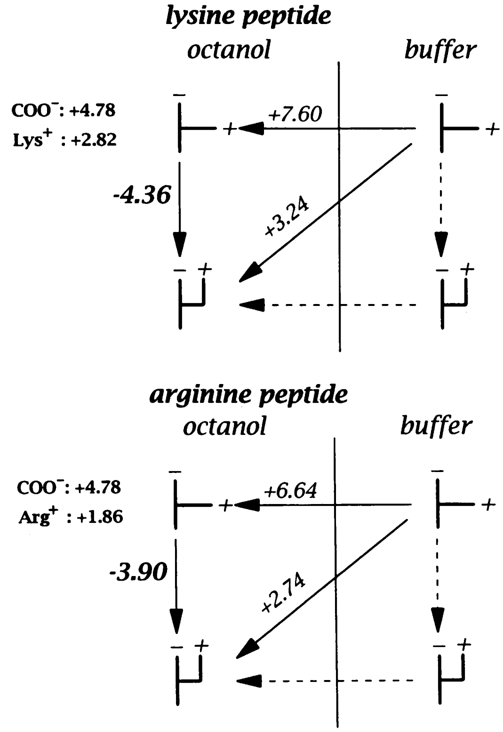

FIG. 5. Estimation of the free energy of salt-bridge formation by using thermodynamic cycles depicting the partitioning of AcWL-K-LL and AcWL-R-LL between water and octanol. Transfer free energies (kcal/mol) are given for each step relative to AcWL-G-LL at pH 1.0. The horizontal steps are the expected water-to-octanol transfer free energies obtained using the pH 1 data of Table 1 under the assumptions that the charges do not interact and that $\Delta G_{COO^-} = 4.78$ kcal/mol (11). The diagonal steps are the experimentally measured values. The differences in transfer free energies, shown as the vertical steps, are due to the intermolecular salt bridge that occurs in the octanol phase between the Arg or Lys side chain and the terminal carboxyl group. The dashed arrows indicate steps of unknown free energy because salt bridges do not form to a measurable extent in the aqueous phase.

thermodynamic analysis shown in Fig. 5. The water-to-octanol free energies of transfer of AcWL-K-LL and AcWL-R-LL that are expected at pH 9 relative to AcWL-G-LL at pH 1 are

$$\Delta G_{\mathrm{exp}} = \Delta G_{\mathrm{COOH}} + \Delta G_{\mathrm{X}},$$

where ΔG_{COOH} is the cost of deprotonating carboxyl group (+4.78 kcal/mol) and ΔG_{X} the free energy cost of transferring the Arg or Lys side chain taken as the pH 1 values in Table 1. The pH 1 values are appropriate because at that pH the side chains are fully charged and there can be no ionic interactions with the fully protonated C terminus. The expected transfer free energies (horizontal arrows in Fig. 5) are clearly much more unfavorable than the observed ones (shown as diagonal arrows). The differences between the expected and observed free energies are the stabilization free energies of the salt bridges: −3.90 kcal/mol for AcWL-R-LL and −4.36 kcal/mol for AcWL-K-LL. The latter value is very close to the value of −4.1 kcal/mol obtained independently from the shift in the $\mathrm{p}K_{\mathrm{a}}$.

We can therefore say with reasonable confidence that the salt bridge reduces the free energy of transferring a charge pair by about 4 kcal/mol. This estimate is based upon our specific knowledge of the interaction of Arg and Lys with the terminal carboxyl group. How does this estimate compare to the cost of transferring any pair of oppositely charged side chains? To answer that question, one must first correct the ionized side-chain transfer free energies of Table 1 for the anomalous transfer free energy of the AcWL-G-LL peptide used as the reference value in Table 1 and for accessible surface area (ASA) occlusion effects that arise from the bulky neighboring side chains of the host pentapeptide.

Ionized-Side-Chain Transfer Free Energies. We have reported (11) values for the octanol-to-water solvation energies of the side chains of the 20 natural amino acids in the X position of the AcWL-X-LL pentapeptides. They are useful in the context of protein folding because the pentapeptides represent a closer approximation to unfolded protein chains than values derived from single-residue model compounds, such as the acetyl amino acid amides (19), due to the presence of neighboring side chains and a longer peptide backbone. An interesting finding that arose in the course of our study was that the AcWL-G-LL peptide was apparently more hydrophobic than the AcWL-A-LL peptide. Because the partitioning of AcWLLL-G and AcWLLL-A behaved normally (i.e., the Ala peptide was more hydrophobic than Gly peptide by an amount expected from the difference in nonpolar ASA), we concluded that the partitioning of AcWL-G-LL was probably anomalous due to conformational effects arising from the presence of the Gly in the center of the chain. Polypeptide solvation energies are generally, and logically, separated into side-chain and backbone contributions with Gly taken as the fundamental backbone unit. We therefore defined a "virtual Gly" transfer free energy based upon the AcWLLL-G/AcWLLL-A measurements that corrected for the conformational anomaly of the AcWL-G-LL peptide. This correction lowers the octanol-to-water transfer free energy of the virtual peptide, AcWL-G*-LL, relative to AcWL-G-LL by 0.62 kcal/mol (11). This means that the magnitudes of the ionized-side-chain $\Delta\Delta G$ values of Table 1 must be reduced by that amount.

Monte Carlo hard-sphere simulations of the ASAs of the AcWL-X-LL pentapeptides revealed that the X residue has a significant effect on the total nonpolar ASA of the peptides and consequently affects the absolute free energies of transfer (11). The effect arises because of changes in the occlusion of the ASA of the guest by the host residues and changes in occlusion of the host residues caused by the guest. Thus, the total nonpolar ASA depends upon which residue is in the X position and one must correct for the resulting changes in the host ASA. The correction typically reduces the magnitude of transfer free energies by about 0.3 kcal/mol so that the final correction to each of the ionized-side-chain $\Delta\Delta G$ values in Table 1 is about 0.9 kcal/mol. The ASA-corrected values (11) of the octanol-to-water transfer free energies for the ionized side chains relative to the virtual Gly peptide are noted in Table 2. The transfer free energies relative to the hydrophobic residue Leu are also given in Table 2.

The values of $\Delta\Delta G$ in Table 2 represent the free energies of transfer of the entire side chain including the methylene fragments and the polar moiety and its ionization. The ionization energies of Asp, Glu, and His can be easily estimated from the pH 1 and pH 9 values (11) of ΔG to be about −3.0, −3.3, and −2.5 kcal/mol, respectively, which, interestingly, are significantly smaller than the value for the C terminus. The ionization energies of Arg and Lys were not determined for technical reasons related to the salt-bridge formation. The ΔG values for the ionized polar moiety contributions of the charged side chains can be estimated by accounting for the hydrophobic contribution of the methylene groups. The values found for Asp, Glu, Arg, Lys, and His are −2.36, −2.80, −1.62, −3.25 and −1.25 kcal/mol, respectively. The Asp and Glu values, and presumably those for Arg and Lys, are smaller than the ionization values for reasons having to do with hydrogen bonding and conformation differences between octanol and water (11). The Arg and His free energies are probably smaller than the Lys value because the charge is distributed over a larger surface area and because of more effective hydrogen bonding with the octanol.

Table 2. Matrices of octanol-to-water free energies of transfer of noninteracting pairs of oppositely charged amino acid side chains relative to Gly or Leu

Relative to		Free energy of transfer, kcal/mol		
Gly		Arg (−0.66)	His (−1.18)	Lys (−1.65)
	Asp (−2.49)	−3.15	−3.67	−4.14
	Glu (−2.48)	−3.14	−3.66	−4.13
Leu		Arg (−3.06)	His (−3.58)	Lys (−4.05)
	Asp (−4.89)	−7.95	−8.47	−8.94
	Glu (−4.88)	−7.94	−8.46	−8.93

Corrected side-chain contributions to the solvation energy of ionizable amino acids are shown in parentheses in kcal/mol (11). The correction procedure is summarized in the text and discussed in detail by Wimley et al. (11).

The free energy gained upon transferring the six possible noninteraction +/− side-chain pairs from octanol to water, as shown in Table 2, range from −3.14 kcal/mol for Glu–Arg to −4.14 for Asp–Lys relative to Gly. If the side-chain pairs interact to form salt bridges in the octanol phase, then the free energy of the pair will be reduced by about 4 kcal/mol. Salt-bridge formation thus approximately cancels the cost of transferring the charge pair to octanol. For comparison, the free energy associated with the transfer of a pair of Leu residues (11) to octanol is −4.8 kcal/mol. The free energy gained relative to Leu when the six possible noninteracting charge pairs are transferred to water, shown in Table 2, range from −7.94 kcal/mol for Glu-Arg to −8.94 for Asp-Lys. Thus, salt-bridge formation can compensate for only about half of the cost of transferring the charge pair when considered relative to Leu.

Implications for Protein Folding. Octanol-to-water transfer free energies of model compounds are frequently utilized in the quantitative analysis of the contribution of solvation energies to protein folding (20–23). Are the salt-bridge solvation energies in Table 2 relevant to protein stability? The *n*-octanol used in partitioning experiments is a well-hydrated liquid phase with a micellar structure (24), whereas folded proteins have a crystal-like interior (25, 26). However, the interior of a folded protein, like octanol, provides opportunities for both hydrophobic and hydrogen-bonding interactions. A simple calculation shows that the concentration of hydrogen-bonding groups in hydrated octanol is ≈11 M while in folded proteins it is ≈25 M. The contribution of hydrophobic solvation of side chains to protein stability, while difficult to separate from conformational and packing effects, is generally found to be similar to the octanol transfer free energies of model hydrophobic compounds (27–31). Despite these indications that octanol partitioning may be relevant to protein stability, we caution that the values of Table 2 are probably not directly applicable for three reasons. (*i*) Electrostatic interactions are sensitive to the apparent dielectric constant of the surrounding medium. The local dielectric coefficient of charged residues can vary between 2 and 78 depending upon the exposure to and/or proximity of bulk water while neat octanol has a value of 10.3 (32). (*ii*) We have not accounted for possible differences in conformational entropy between the peptide system and folded proteins. (*iii*) We do not know how the conformations and interactions of the octanol itself contribute to the free energy of partitioning. However, despite these differences between octanol and the interiors of proteins and the uncertainties of applying octanol partitioning data to the protein folding problem, our measurements agree quantitatively with the calculations of Hensch and Tidor (10) for salt bridges in proteins that are 70% buried.

Salt bridges in proteins that are stabilizing provide less stability than hydrophobic residues packed equivalently (8). Importantly, in their combinatorial mutagenesis studies, Waldburger *et al.* (8) never recovered active variants of the Arc repressor, other than the wild-type itself, that conserved a stabilizing salt-bridged triad; hydrophobic residues replaced the triad in all of the active and partially active variants. These results and our finding that salt bridges are energetically neutral in octanol partitioning suggest that, on average, buried salt bridges are energetically neutral in terms of protein stability and consequently will provide less stabilization than buried hydrophobic residues. What do our results say about the accuracy of computational and theoretical studies of salt bridges in proteins? Bearing in mind the likely structural dependence of salt-bridge energetics in proteins and the uncertainties inherent in comparisons of octanol data with folding data, our findings are in excellent agreement with free energies obtained in computational studies. Hendsch and Tidor (10) typically found that the free-energy decrease associated with salt-bridge formation was about 50% smaller than the increase associated with desolvation of the pair, calculated relative to hydrophobic isosteres, and this is exactly the result we obtain in our model system. A similar estimate was obtained by Honig and Hubbell (33) who estimated that the free energy cost of forming an ion pair in a medium with dielectric constant 10 would be about 5 kcal/mol.

Our present results, the site-directed mutagenesis studies of Waldburger *et al.* (8), and the computational studies of Hendsch and Tidor (10) agree: Salt-bridged charge pairs do not, and probably cannot, provide as much stabilization to proteins as a pair of hydrophobic residues of about the same volume. The remarkable agreement between the three studies suggests that protein electrostatic computational methods have come of age.

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