Strength of Solvent-Exposed Salt-Bridges†

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This paper uses a recently developed computer model to study the energetics of solvent-exposed salt-bridges. The model uses the "mining minima" method to compute conformational free energies with the CHARMm empirical force and the generalized Born solvation model. Satisfactory agreement is obtained in comparison with the measured binding affinities of ion pairs in solution and with the salt-bridge energetics deduced from studies of salt-bridges in helical peptides. The calculations suggest that stabilizing charge−charge interactions in helical peptides do not require well-defined salt-bridge conformations. This is in agreement with crystallographic studies of charge pairs added to T4 lysozyme by site-directed mutagenesis. The computer model is also used to make a testable prediction that arginine and phosphotyrosine residues in an (i, $i + 4$) relationship will form a particularly strong salt-bridge in helical peptides. The biological implications of these results are discussed.

Salt-bridges have long been regarded as contributors to the stability and function of proteins.[1] Perhaps the classic example is the allosteric transition of hemoglobin, in which a number of salt-bridges are believed to stabilize the T state and to break or become weaker in the R state, thus contributing to the cooperativity of the allosteric transition.[1] Furthermore, heat-stable proteins from thermophilic organisms tend to have more salt-bridges than their mesophilic counterparts,[2−9] and deletion of salt-bridges can destabilize the native states of proteins.[7,10−12] Salt-bridges between oppositely charged side chains in an (i, $i + 4$) sequence relationship also have been shown to stabilize the helical forms of peptides.[13−16]

At the same time, it has become clear that one cannot with confidence rely on a salt-bridge to be stabilizing. Thus, the introduction of a partly buried salt-bridge into the interface of a dimer of α-helices destabilized the dimer by about 8.0 kJ/mol.[17] Another experimental study involved two arginines and a glutamic acid that form a salt-bridging triad in a solvent-sequestered location in the Arc repressor homodimer; when these residues were replaced with hydrophobes, the protein became more stable by 16.0 kJ/mol.[18] (It is worth noting, however, that this experiment deleted not only a net-neutral ion pair but also an additional +1 charge from the protein interior. Neutralization of a buried charge is known to be net stabilizing on the basis of both theory[19] and experiment.[20−22]) Furthermore, a study of the energetics of a salt-bridge, where octanol was used as the model of the protein interior, concluded that the "buried" salt-bridge was net destabilizing relative to the interaction of a pair of neutral isosteres.[23] Results of this type had actually been anticipated by a computational study of the energetics of salt-bridges[24] that used the PB model[25−27] of protein electrostatics.

The same computational study found that the two most solvent-exposed salt-bridges examined were net stabilizing by about 4.0 kJ/mol.[24] This result, combined with experimental data on the stabilization of α-helices by salt-bridges mentioned above, seems to suggest that solvent-exposed salt-bridges might, indeed, be relied upon to stabilize proteins, as suggested by double-mutant studies of ionic interactions in barnase.[28] However, a counterexample exists for even this limited attempt to establish rules for the energetics of salt-bridges. Thus, the use of site-directed mutagenesis to add oppositely charged groups in T4 lysozyme in solvent-exposed positions that might permit them to form salt-bridges led to little if any net stabilization of the native state.[29]

It should be emphasized that the various studies reviewed above do not necessarily contradict each other. For one thing, they do not all express the energy of a salt-bridge relative to the same reference state, so different studies may be reporting different quantities. This issue has been discussed previously[24] and is considered further in the Discussion of the present paper. Another important reason for the varied picture of salt-bridge energetics is that the energetics of a salt-bridge depends on what other chemical groups are nearby, on solvent conditions, and on temperature. Predicting whether a given salt-bridge will indeed be stabilizing is difficult, but the ability to do so would be useful in understanding naturally occurring protein structures and in redesigning proteins to be more stable.

Computational methods may be useful in making such predictions. Indeed, significant insight has already been gained from the application of classical electrostatics models to this problem.[24,30] However, these studies have modeled the protein as an essentially rigid structure capable of adopting only one or two different conformations. This restriction results chiefly from the fact that solving the Poisson−Boltzmann (PB) equation in detail for a large number of molecular conformations is

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prohibitively time-consuming. Moreover, even given a method for solving the PB equation quickly for many conformations, it would not be immediately clear how to apply it to the problem of salt-bridge energetics.

Recently developed computational methods make it possible to address some of these issues in at least a preliminary fashion. The efficient generalized Born (GB) solvent model[31,32] has been shown to yield good agreement with finite difference solutions of the Poisson equation. The computational speed afforded by the GB model allows a reasonable electrostatic model to be used in calculations that account for conformational flexibility. A recently developed conformational analysis method is particularly useful for free energy analyses. This "mining minima" (MM) method[33] computes the conformational free energy of a molecule by locating stable conformations and computing the free energy of each one with a Monte Carlo integration method. The overall free energy is then computed via a sum over the various stable conformations. This method has been shown to yield convergent results for stretches of polyalanine of up to 10 residues and for a flexible inhibitor of HIV protease.[33]

The MM and GB methods have already been used together to compute the pK_as of a series of dicarboxylic acids and amino acids and of the active-site aspartyl dyad in HIV-1 protease.[34] The results agree rather well with experimental results for the small molecules. The results for HIV protease are less accurate but do yield reasonable agreement with experimental results. These results suggest that the MM/GB method may be used to compute salt-bridge energetics while allowing for conformational flexibility, at least for simple systems.

The present paper thus uses the MM/GB method to compute the energetics of solvent-exposed ion pairs. Several different systems are examined. First, the binding affinities of guanidinium and ammonium compounds with carboxylates and phosphates in solution are computed and are compared with experimental results.[35] Then the contributions of salt-bridges between histidine and aspartic acid side chains to the stability of α-helix-forming peptides are computed and compared with experimental results.[15] These validation studies of ion-pairing yield results that agree with experimental free energies to within several kJ/mol. In particular, calculations and experiment agree that solvent-exposed ion-pairs between monoionic groups are stabilizing, but only weakly. The calculations show, furthermore, that the side chains can remain quite mobile, even when their interaction stabilizes the helical conformation of the peptide.

The present model allows us a number of structural and energetic issues raised in previous studies to be addressed in detail. Furthermore, we use the model to identify an ion pair stronger than those so far examined. To this end, the MM/GB method is used to compute the stabilization afforded by salt-bridges involving naturally occurring dianionic side chains. The calculations indicate that a salt-bridge between arginine and phosphotyrosine is likely to be about twice as strong as the surface salt-bridges that have been studied experimentally in helix-forming peptides to date. This testable prediction is particularly interesting because arginine−phosphotyrosine salt-bridges are known to be important in cell signaling pathways.[36,37]

Methods

This section details the computational methods used in the present study. The first part describes the molecular systems and their representation in the calculations. The second and third parts describe the calculation of relative α-helix stabilities and of the binding affinities of ion pairs in solution. The fourth part describes the potential energy and solvation energy models. The final part reviews the experimental studies used for validation.

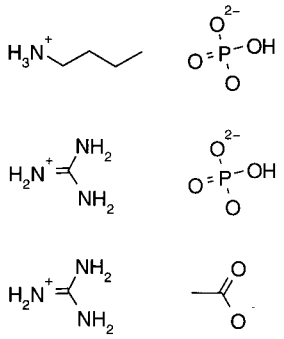

Figure 1. Free ion pairs studied in this paper.

TABLE 1: Sequences of Modeled Helix-Forming Peptides

peptide	sequence
HD3	Ac-AAAAA**H**AA **D**AAAAAAAAA-NH_2
HD4	Ac-AAAAA**H**AAA **D**AAAAAAAA-NH_2
HD5	Ac-AAAAA**H**AAAA **D**AAAAAAA-NH_2
DH3	Ac-AAAAAAA**D**AA **H**AAAAAAA-NH_2
DH4	Ac-AAAAAA**D**AAA **H**AAAAAAA-NH_2
DH5	Ac-AAAAA**D**AAAA **H**AAAAAAA-NH_2

Molecular Models. Three ion pairs are examined here: butylammonium with HPO_4^{2-}, guanidinium with HPO_4^{2-}, and guanidinium with acetate (Figure 1). The molecules are built with Quanta 4.1[38] from standard molecular fragments. The binding energy calculations are carried out with the molecules fixed in their ideal initial conformations.

A series of six peptides with histidine and aspartate side chains are studied. These model ones previously studied experimentally.[15] Three of the peptides possess a histidine and an aspartate in (i, $i + 3$), (i, $i + 4$), and (i, $i + 5$) relationships and are named, respectively, HD3, HD4, and HD5. The other three peptides are the same except that the order of the histidine and the aspartate is reversed. These are named DH3, DH4, and DH5. In the experimental studies, the peptides also possess two lysine residues, a glycine and a tyrosine. However, these are located near the termini and far from the histidine and aspartate residues. Here, all residues other than histidine and aspartate are modeled as alanines for simplicity. The helices are capped with neutral terminators both here and in the experimental studies. The resulting sequences are listed in Table 1. The experimental studies were carried out at pH 2, 5.5, and 9 to force the histidine and aspartate residues into a series of different protonation states. To simulate these conditions, calculations are done here with histidine and aspartate in the protonation states appropriate to these three pH values; i.e., histidine ionized/aspartate neutral, histidine ionized/aspartate ionized, and histidine neutral/aspartate ionized. The peptides are built as polar-hydrogen-only ideal α-helices with Quanta 4.1.[38] The geometry of the two terminal caps is idealized by up to 200 steps of conjugate gradient energy-minimization with only covalent bond terms. The MM calculations treat the entire helix as rigid: only the rotatable side chain bonds of the histidine and aspartate are allowed to vary. The atoms of these side chains are treated in full detail, but the helix, including all the alanine side chain C_β atoms, is modeled as a "dummy" that interacts with the side chains with only van der Waals repulsions.

To search for stronger salt-bridges, calculations are also done for three additional series of peptides that have not been studied experimentally. These are similar to the histidine−aspartate series described above, but different side chains are examined. The side chains are lysine with phosphotyrosine, arginine with

phosphotyrosine, and arginine with dianionic γ-carboxyglutamic acid. These peptides are built in the same way as the HD series above and are named as follows: KY_p3, KY_p4, KY_p5, RY_p3, RY_p4, RY_p5, $RE_\gamma3$, $RE_\gamma4$, and $RE_\gamma5$. Here, Y_p is phosphotyrosine and E_γ is γ-carboxyglutamic acid. The reverse-order peptides (see previous paragraph) are not examined here. However, the calculations are done with the phosphate in the monoionized (1-) and doubly ionized (2-) states.

Computation of Relative Stabilities of α-Helices. The experimental studies of the histidine−aspartate peptides use circular dichroism (CD) to measure the fractional helicity of peptides as a function of the sequence gap *n* between the charged groups. The reported salt-bridge energies are referenced to the peptide with a sequence gap of 5, (i, $i + 5$), and it is assumed that *n* does not influence the free energy of the coil state.[15] The present calculations use the same assumption and the same reference state. This makes it unnecessary to compute the free energy of the coil state of the peptide, a task that would be difficult computationally. It is only necessary to compute the chemical potentials of the various peptides in their helical conformation, relative to the chemical potential of the (i, $i + 5$) peptide.

The chemical potential of the helical form of the (i, $i + n$) peptide relative to that of the (i, $i + 5$) peptide can be written in terms of classical configuration integrals Z_n:

$$\Delta\mu^\circ = -RT \ln\left(\frac{Z_n}{Z_5}\right) \tag{1}$$

$$Z_n \equiv \int \mathrm{e}^{-(U_n(\mathbf{r})+W_n(\mathbf{r}))/RT}\, d\mathbf{r} \tag{2}$$

where R is the gas constant, T is the temperature, $U_n(\mathbf{r})$ and $W_n(\mathbf{r})$ are, respectively, the gas-phase potential energy and solvation energy of the helical (i, $i + n$) peptide as a function of its conformation, and $\mathbf{r}$ is a vector of internal coordinates that specifies the conformation. Here, the configuration integrals extend over the rotatable torsions $\boldsymbol{\theta}$ of the side chains, but the peptide is otherwise treated as a rigid helix. The MM method[33] computes these integrals by summing contributions from low-energy conformations. The computed relative chemical potentials are compared directly with the corresponding experimental values.

The average configurational energy of the helical form of the (i, $i + n$) peptide can be written as

$$\langle U_n + W_n \rangle = \frac{\int (U_n(\mathbf{r}) + W_n(\mathbf{r}))\, \mathrm{e}^{-(U_n(\mathbf{r})+W_n(\mathbf{r}))/RT}\, \mathrm{d}\mathbf{r}}{Z_n} \tag{3}$$

where Z_n is defined in eq 2. The MM method computes this average energy directly at the same time as it computes the free energy.

The MM calculations are judged to be converged when the computed chemical potential does not change by more than 1 part per million when the contribution of a new energy-minimum is added. Convergence is readily achieved for these systems. This is illustrated in Figure 2, which plots cumulative free energy versus number of minima accumulated for three peptides HD3, HD4, and HD5. Interestingly, achieving convergence for HD4 requires fewer minima than for HD3 and HD5, although all three systems have the same number of degrees of freedom. This indicates that the free energy of HD4 is dominated by a few important energy minima, while the energy landscapes of HD3 and HD5 are smoother. Table 2 lists serial computer timings for the six histidine−aspartate peptides.

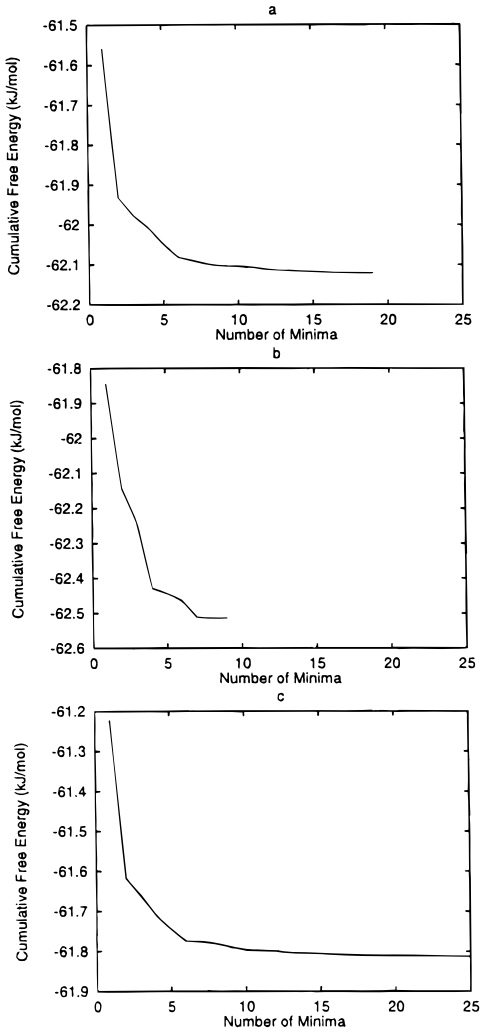

Figure 2. Convergence of free energy calculations for helical peptides HD3 (a), HD4 (b), and HD5 (c) at pH5.5.

TABLE 2: Numbers of Energy-Minima (*M*) and CPU Times (SGI R10000 Indigo 2) Required To Compute Converged Free Energies for Peptides at pH 5.5

peptide	*M*	time (s)
HD3	19	5100
HD4	9	2500
HD5	25	7400
DH3	30	8800
DH4	8	2900
DH5	36	10100

The helix calculations with lysine and arginine side chains require integration over 8−10 torsional degrees of freedom. These calculations are executed with a parallelized version of the MM software that is implemented with the message-passing interface (MPI) standard and run on four nodes of an IBM SP-2 computer. A speedup of about 3.5 over single-node sequential calculations is achieved. As a consequence, the wall-clock time is 7 h or less for all MM calculations presented in this paper.

To examine the mobility of the salt-bridged histidine and aspartate side chains, a Metropolis[39] Monte Carlo calculation is carried out for the HD4 peptide. As for the MM calculations, the side chain torsion angles are the only degrees of freedom in this calculation. The dihedral step-size is adjusted to yield an acceptance rate of about 50% for trial conformations, and about 250 000 accepted conformations are generated. Every 20th one of the accepted conformations is used to compute the mean positions of the atoms and their mean-square deviations from

these means. These running averages become constant to within 0.2 Å^2 once the first few hundred conformations are included.

Computation of Binding Free Energies of Ion Pairs. The standard free energy of binding ΔG° for molecules A and B equals the change in chemical potential under hypothetical conditions of ideality and standard concentration:[40]

$$\Delta G^\circ \equiv \mu^\circ_{AB} - \mu^\circ_A - \mu^\circ_B \tag{4}$$

where μ°_x is the standard chemical potential for species x, with x = A, B, and AB. Writing these standard chemical potentials in terms of configuration integrals yields the following expression for the standard free energy of binding:[40]

$$\Delta G^\circ = -RT \ln \frac{Z_{AB}}{Z_A Z_B} + RT \ln \frac{8\pi^2 \sigma_{AB}}{C^\circ \sigma_A \sigma_B}$$

where σ_x is the symmetry number of species x and C° is the standard concentration, which is taken to be 1 mol/L here. The configuration integrals for molecules A and B are of the form

$$Z = \int e^{-(U+W)/(RT)}\, d\boldsymbol{\theta} \tag{5}$$

where, as above, the integral ranges over only torsion angles. The configuration integral for the complex Z_{AB} extends not only over the torsion angles $\boldsymbol{\theta}$ of the individual molecules but also over the position $\mathbf{R}_B$ and orientation ξ_B of molecule B relative to molecule A. This configuration integral includes an index function $I(\mathbf{R}_B)$ that defines the complexed state:[40−42]

$$Z_{AB} = \int I(\mathbf{R}_B)\, e^{-(U+W)/(RT)}\, d\boldsymbol{\theta}\, d\mathbf{R}_B\, d\xi_B \tag{6}$$

In the present calculations, $I(\mathbf{R}_B)$ is a step function that is unity when the central atom of molecule B is within a 24 Å × 24 Å × 24 Å box centered at the central atom of molecule A. To permit the evaluation of Z_{AB}, the current implementation of the MM algorithm samples isotropically over the position and orientation of one molecule with respect to the other. This procedure automatically samples the multiple conformations of the complex that are the most stable, as described in Results. A complete binding calculation for the molecules considered here requires 2−4 h of processor time on an SGI R10000 workstation. All calculations use a locally modified version of the program UHBD.[43]

Energy Model. As noted above, the energy in the configuration integrals can be separated into a potential energy $U(\mathbf{r})$ and a solvation energy $W(\mathbf{r})$.[40] Here, the CHARMm 22.0 empirical force field with polar hydrogens only is used for the potential energy.[44] Parameters are assigned with the program Quanta 4.1[38] and, where necessary, the ChemNote module of Quanta. A molecular dielectric constant of 2 is used to account for electronic polarizability,[45] as in modeling polar substituent effects. The GB model[31,32] is used for W. No solvation contribution related to molecular surface area is included in W because the surface area of the systems studied here vary little with conformation. The GB solvation energies are evaluated with the radius of each atom set to the mean of its CHARMm $\boldsymbol{\sigma}$ parameter and that of water (atom type OW) except that hydrogen radii are set to 1.2 Å. The solvent dielectric constant is set to 78.

In the experimental study of salt-bridges in helix-forming peptides, the ionic strength was only 10 mM. As a consequence, the neglect of ionic screening in the GB model used here should not be a significant problem. However, in the experimental study of ion association, the measurements were done in a solution of high (1 mol/L) ionic strength. This is expected to influence the binding free energy of charged groups significantly. Therefore, the computed binding affinities are adjusted for ionic strength by a series of FDPB calculations. For each ion pair, the lowest energy-bound conformation is taken as a model of the complex and the FDPB method is used to compute the solvation energies of the complex and the separated species, both with and without 1 M salt. This corresponds to six FDPB calculations. The change in solvation energy upon complexation in the presence of salt is subtracted from that without salt. The difference is an estimate of the influence of salt upon the binding energy, and this is added to the binding energies computed with the MM/GB method. The ionic strength corrections are on the order of 4.0 kJ/mol, opposing binding.

Experimental Data. The experimental study of ion−ion association referred to here detects association via pK_a shifts,[35] and extracting free energies of association from the measurements involves curve fitting. The standard deviations of replicated measurements of the acid dissociation constants are reported at roughly 0.03−0.26. No experimental uncertainties are provided for the computed ion association constants, but it is stated that these uncertainties are "considerably greater" than those for the acid dissociation constants because the fitted curves do not match the experimental data perfectly. Also, the measurements are carried out at a constant 1 mol/L ionic strength to determine association constants independent of ionic strength. The ionic strength was maintained over various concentrations of the ions of interest by the addition of tetramethylammonium (TMA) chloride. It is stated that these added ions do not complex significantly with the ions of interest—guanidinium, HPO_4^{2-}, $H_2PO_4^{1-}$, and butylammonium—but the basis for this statement is not provided. Our test calculations suggest that TMA does bind HPO_4^{2-} and acetate with free energies of about −1.5 and −4.0 kJ/mol, respectively. As a consequence, we believe that the reported binding energies might be in error by 1.0−4.0 kJ/mol.

The contributions of salt-bridges to the stability of α-helices with oppositely charged side chains in an (i, $i + n$) sequence relation are evaluated experimentally by using CD to evaluate the change in fractional helicity with n.[46−48] The CD data are interpreted with the Lifson−Roig theory of helix−coil equilibrium, augmented by terms accounting for helix-dipole electrostatics, "capping" of the peptide termini, and side chain interactions.[49−53] The reported salt-bridge energies are given without estimates of uncertainty. However, it is stated that salt-bridges worth less than 0.6 kJ/mol would not be detected; this gives at least some sense for the reliability of the results. It is also worth noting that the salt-bridge energies that are reported are model-dependent quantities; any inadequacy in the modified Lifson−Roig theory might well influence the reported energies. In addition, the extraction of fractional helicities from CD measurements is subject to error. For example, as recently emphasized,[54] circular dichroism does not clearly differentiate the α-helix from the 3_{10} helix. If the peptides studied here were to adopt alternative helical conformations, the interpretation of the data in terms of a two-state α-helix−coil equilibrium might lead to errors in the reported salt-bridge energetics. Similarly, the experimental interpretation (as well as our calculations) assumes that the salt-bridges do not contribute to the stability of the coil state of the peptides. There is some uncertainty regarding the degree to which this assumption may introduce error.[30]

It is worth noting that the study of helix-forming peptides also reports the pK_as of the histidine and aspartate in the various

TABLE 3: Binding Free Energies (kJ/mol) of Ion Pairs[a]

ion pair	ΔG°_{calc}	ΔG°_{expt}
guanidinium−phosphate	−4.10	−2.38
butylamine−phosphate	−4.01	−1.76
guanidinium−acetate	−3.26	2.51

[a] Phosphate is dianonic here. ΔG°_{calc}, calculated; ΔG°_{expt}, measured.[35]

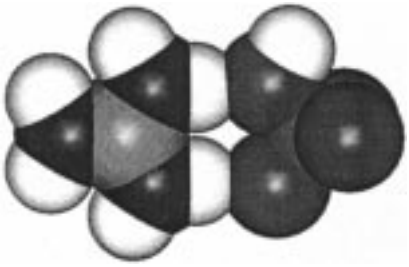

Figure 3. Low-energy conformation of guanidinium and phosphate.

peptides. Although it would be of interest to try computing these pK_as, such calculations would require modeling the coil state of the peptide. This is because the peptides studied are only 15−40% helical; thus, the pK_as are largely determined by coil conformations.

Results

Binding Affinities of Ion Pairs. Table 3 presents computed and experimental standard free energies of binding for the three ion pairs examined here. The computations agree with experimental results in the following respects. First, the magnitudes of the binding affinities are all within a few kJ/mol of zero, as found experimentally. Second, the affinities of the three ion pairs are ranked correctly, with the guanidinium−phosphate pair most stable. Third, both computation and experiment indicate that the guanidinium−phosphate and butylamine−phosphate pairs have similar binding affinities. On the other hand, the computed binding affinities are all somewhat stronger than those found experimentally, especially in the case of guanidinium−acetate. The basis for this trend is not clear. However, as discussed in Methods, the experimental data may be subject to systematic errors due to association of TMA and/or Cl^- with the reactants. Overall, then, the agreement with experiment is satisfactory.

The MM method provides not only the overall free energy of the ion-pair complexes but also the atomic coordinates and free energies of all stable conformations of each complex that are identified during the calculation. The calculations indicate that the most stable conformations of the guanidinium−phosphate complex have twin hydrogen bonds from two phosphate oxygens to two neighboring guanidinium hydrogens (Figure 3). These conformations are similar to those observed in relevant crystal structures of proteins.[37] That the MM procedure automatically discovers the energetically important conformations of the complex is highlighted in Figure 4. This shows the guanidinium along with a dot indicating each sampled position of the phosphorus atom. The dots cluster appropriately at the three equivalent sites where the optimal doubly hydrogen-bonded complex can form. Examination of the conformations generated by the MM algorithm shows that it also includes all relevant orientations of the phosphate relative to the guanidinium.

Although the binding affinities for these ion pairs are near zero, the complexes bind in a pronounced energy ($U + W$) well. For guanidinium−phosphate, this energy well is about 16.0 kJ/mol deep, as shown in Figure 5. The existence of a sharp energy well for the bound complex means that the precise definition

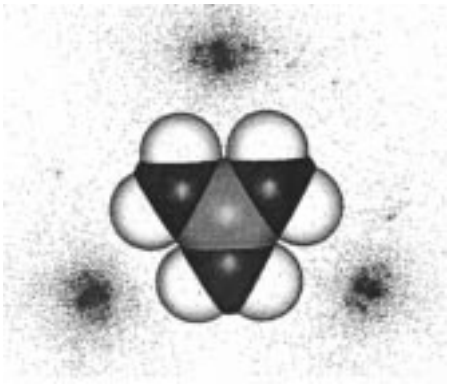

Figure 4. Sampling of phosphate positions around guanidinium. Each dot represents a position of the phosphorus atom during the hunt for low-energy conformations.

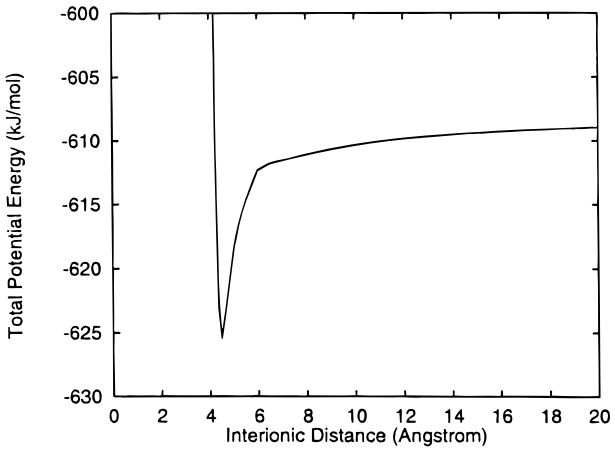

Figure 5. Total configurational energy, $U + W$, of the guanidinium−phosphate complex versus the distance between the guanidinium carbon and the phosphate phosphorus atoms as the complex shown in Figure 3 is pulled apart along the carbon−phosphate axis. The orientation of the two ions is held fixed.

of the complex will not strongly influence the computed binding free energy, as previously discussed.[40]

A separate experimental study[55] estimated the maximal contribution of a guanidinium−phosphate interaction to the binding affinity of a host−guest pair at about −10.0 kJ/mol. This estimate was made by comparing binding affinities of host−guest complexes with and without a guanidinium−phosphate interaction. The model used to extract this estimate from the data subtracts off changes in translational and rotational entropy, changes in entropy associated with the restriction of two rotatable bonds, and changes in strain and noncovalent interaction energy associated with other parts of the host−guest complex. Therefore, in the notation of the present paper, the maximal contribution of the guanidinium−phosphate interaction corresponds to the change upon binding in the part of $\langle U + W \rangle$ that is associated with the guanidinium−phosphate contact. (The angle brackets here indicate a thermodynamic average.) Because the MM method computes configuration integrals, it allows the change in $\langle U + W \rangle$ to be calculated directly for the guanidinium−phosphate ion pair. The result is −10.7 kJ/mol, in excellent agreement with the prior estimate. Note that this value is less negative than the −16.0 kJ/mol depth of the energy well (Figure 5) because the complex is not frozen at the very bottom of the well; it also occupies conformations of higher energy because of thermal agitation, and this makes the average energy less negative.

TABLE 4: Histidine−Aspartate Interaction Energies (kJ/mol) in a Helical Peptide for Three pH Values[a]

pH	peptide	ΔG_{calc}	ΔG_{expt}
pH 2.0	HD3	−0.50	> −0.63
	HD4	−1.25	−2.38
	DH3	−0.67	> −0.63
	DH4	−0.92	> −0.63
pH 5.5	HD3	−1.30	−2.55
	HD4	−2.93	−3.14
	DH3	−1.25	> −0.63
	DH4	−2.26	> −0.63
pH 9.0	HD3	−0.46	−1.88
	HD4	−0.54	> −0.63
	DH3	−0.33	> −0.63
	DH4	−0.29	> −0.63

[a] As in the experimental study, results for HD3 and HD4 are relative to HD5 and results for DH3 and DH4 are relative to DH5, at each pH. Values of "> −0.63" are those not included in the experimental table of results. The cutoff of −0.63 results from the report that interactions would not be detected if they were one-third as strong, combined with the fact that the weakest listed interaction is −1.88 kJ/mol.[15]

Influence of Histidine−Aspartate Interactions upon α-Helix Stability. Table 4 presents computed and measured side chain interaction energies. The computations agree with experiment in several important respects. First, the helix stabilizations afforded by the histidine−aspartate interaction are of the correct order of magnitude, i.e., from about zero to about 3.0 kJ/mol. Second, the greatest stabilization is correctly ascribed to the HD4 peptide at pH 5.5. Third, the stabilization for the two HD peptides is correctly found to be greatest at pH 5.5, at which both side chains are ionized. Fourth, the HD4 interaction is found to be significantly stabilizing at pH 2, even though the aspartate is neutral at this pH.

On the other hand, the computations fail to reproduce two unexpected results that were highlighted in the experimental paper. First, it was observed that the histidine−aspartate interactions in HD3 and HD4 at pH 5.5 were stabilizing relative to HD5, but the interactions were no longer stabilizing when the two groups were swapped to form peptides DH3 and DH4. The calculations fail to reproduce this asymmetry; instead, they suggest that the histidine−aspartate interaction in DH3 and DH4 is stabilizing relative to DH5. Second, the experiments show that the histidine−aspartate interaction in HD3 is stabilizing at pH 9, relative to HD5, even though there can be no salt-bridge at this pH. In contrast, the calculations suggest that the histidine−aspartate interaction in HD3 is scarcely stabilizing relative to HD5. These discrepancies between calculation and experiment could result from a weakness in our model. For example, we do not consider the influence of the histidine−aspartate gap on the chemical potential of the coil state of the peptides. The discrepancies could also result from complexities in the physical system that are not accounted for adequately by the models and assumptions used to extract salt-bridge energies from the experimental data, as discussed above. For example, like our calculations, the model used by Huyghes−Despointes and Baldwin to derive salt-bridge energies from helicity data neglects changes in the coil state.[15]

It is of interest to inquire how strongly the stabilization afforded by these salt-bridges depends on electrostatic versus van der Waals interactions. It is conceivable that the Coulombic attraction between the His and Asp is fully compensated by the loss of solvation energy incurred when the groups are in contact. In this case, electrostatics would make no net contribution to the energy of the salt-bridge. It is even possible that desolvation outweighs attraction and that electrostatics is net destabilizing. This question is addressed by repeating the calculations for HD4 and HD5 with all partial charges set to zero; the remaining attraction between the two groups results entirely from Lennard-Jones forces. These calculations in the absence of charge−charge interactions yield that the HD4 helix is −1.88 kJ/mol more stable than the HD5 helix, somewhat less than the computed stabilization of −2.93 kJ/mol afforded by the ionized histidine and aspartate at pH 5.5. Thus, the present calculations suggest that electrostatics are net stabilizing in this system.

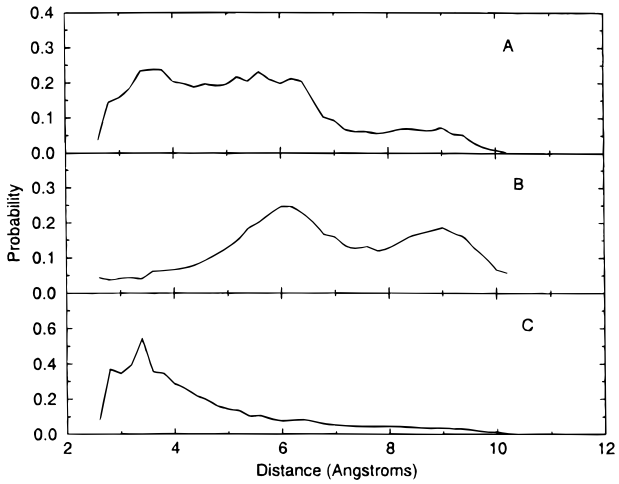

Figure 6. (A) Computed raw probability distribution of distance between the histidine Nδ1 nitrogen and the aspartate Oδ1 oxygen of peptide HD4. (B) Same probability distribution in the absence of nonbonded forces. (C) Probability distribution of distance between the same pair of atoms, corrected by the distribution in the panel B (see text).

The experimental study of Dao-Pin et al. attempted to introduce new salt-bridges into T4 lysozyme by site-directed mutagenesis. Three of the six mutations did stabilize the native state somewhat, but crystallographic analysis of the mutants showed that the charged side chains that had been added were quite mobile, with *B*-factors of 40−70 Å^2. These high mobilities suggested that the charged side chains are not truly salt-bridged and therefore that the weak stabilization they afford should not be attributed to their mutual attraction but to some other interaction. Dao-Pin et al. emphasize the thermodynamic significance of this mobility, noting that forming a tight salt-bridge would require that the configurational entropy of the side chains be sacrificed. The computer model used here allows us to examine these issues for the histidine−aspartate helical peptides.

The mobility of the side chains of HD4 is examined here with a Metropolis Monte Carlo calculation, as described in Methods. This calculation yields a Boltzmann distribution of side chain conformations, which is used to compute the mean positions and mean square deviations of the histidine Nδ1 nitrogen and the aspartate Oδ1 oxygen. The mean square deviations are found to be 3.3 and 1.7 Å, respectively. These correspond to crystallographic *B*-factors of 83 and 45, respectively, which are similar to those observed experimentally for the charged side chains in the T4 lysozyme study[29] (see above). However, despite the large *B*-factors, the histidine and aspartate side chains examined here do spend a significant amount of time in proximity to each other. This is evident from the computed probability distributions of distances between the histidine Nδ1 nitrogen and the aspartate Oδ1 oxygen (Figure 6). The raw distribution of distances, $P(r)$ shows a tendency of the two atoms to be near each other (top graph). However, this distribution is biased by the underlying probability distribution

for the side chains that would exist in the absence of electrostatic and van der Waals interactions. This underlying probability distribution $P'(r)$ is biased toward longer His−Asp distances, as shown in the middle graph of the figure. When the actual probability distribution, $P(r)$, is corrected for the underlying distribution $P'(r)$, the resulting distribution, which is proportional to $P(r)/P'(r)$, shows a distinct bias toward short interatomic distances, as shown in the lower graph of Figure 6. Thus, the mutual attraction of the two groups does tend to pull them together and does stabilize the helical conformation, in comparison with the HD5 peptide. Nonetheless, the two groups do not form a structurally well-defined salt-bridge.

Dao-Pin et al. also pointed out that forming a salt-bridge between flexible side chains incurs an entropic penalty. Thus, stronger salt-bridges might be expected between side chains that are preorganized by the rest of the protein. The present model allows us to estimate the strength of the His−Asp salt-bridges in the case where the side chains are not free to move. We can compute the strength of such a salt-bridge by comparing the point energies ($U + W$) of the optimal conformations of the HD4 and HD5 peptides. The difference is found to be −5.0 kJ/mol, which is 2.0 kJ/mol more stabilizing than the free energy change when mobility is included (see Table 4). A somewhat larger value would be obtained if the side chains were not allowed to relax to their optimal conformations in the (i, $i + 5$) helix. A value of about −5.0 kJ/mol for the stabilization afforded by a rigid surface salt-bridge agrees well with that found by Hendsch and Tidor using a closely related computer model.[24]

Stronger Salt-Bridges Involving Dianions. The salt-bridges considered in the previous section are quite weak: the strongest provides a stabilization that is scarcely greater than *RT* thermal energy. Such salt-bridges would be of little use in molecular design projects aimed at enhancing the stability of a desired conformation. It is therefore of interest to seek stronger salt-bridges that could be used in such applications. The computational method used here agrees well enough with experimental results such that it is reasonable to use it in a predictive mode to check the stabilities of possible strong salt-bridges in helix-forming peptides.

We conjectured that increasing the charge of one or both ions would increase the strength of the salt-bridge. To generate testable predictions, we consider two naturally occurring dianionic side chains that should be accessible experimentally: phosphotyrosine and γ-carboxyglutamic acid. The former is important in intracellular signaling pathways; the latter is involved in Ca^{2+} binding in blood-clotting proteins. Here, these dianionic side chains are paired with either lysine or arginine cations. Calculations are done with phosphotyrosine in both the 1- and 2-protonation states. Table 5 presents predicted side chain interaction energies for the resulting salt-bridges in hypothetical helix-forming peptides.

The most striking result is the prediction that the arginine−phosphotyrosine^{2-} salt-bridge in an (i, $i + 4$) sequence relationship stabilizes the helix by 6.8 kJ/mol, or about 2.5*RT*. This salt-bridge would be over twice as strong as the strongest histidine−aspartate salt-bridge examined in the previous section. Interestingly, replacing arginine with lysine significantly weakens this salt-bridge even though the net charges are unaffected. This contrasts with the results for ion pairs in solution, where butylammonium and guanidinium bind almost equally well to phosphate (see above). The difference results from the constraints imposed by the helix, which prevent the lysine−phosphotyrosine pair from forming optimal hydrogen bonds. Even for the arginine−phosphotyrosine pair (Figure 7), the best hydrogen bonds formed are not as good as those formed between free guanidinium and phosphate (Figure 3).

TABLE 5: Lysine−Phosphotyrosine, Arginine−Phosphotyrosine, and Arginine−γ-carboxyglutamate Interaction Energies (kJ/mol) in a Helical Peptide for Different Charge Status of the Acid[a]

charge status	peptide	ΔG_{calc}
−1	KY_p3	−0.84
	KY_p4	−2.63
	RY_p3	−2.17
	RY_p4	−4.64
−2	KY_p3	−0.96
	KY_p4	−3.64
	RY_p3	−4.31
	RY_p4	−6.77
	$R_{\gamma-CO_2}3$	−3.43
	$R_{\gamma-CO_2}4$	−3.72

[a] Charge status: charge of the interacting acid. See previous table and text for explanations. Only Y_p is examined in its monoanionic form because the salt-bridges with $E_{\gamma-CO_2}$ are already rather weak in its dianionic state.

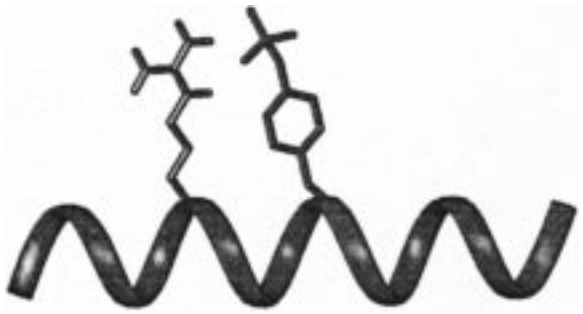

Figure 7. Low-energy conformation of peptide RY_p4 when phosphotyrosine has charge of −2.

It is also of interest that the salt-bridges involving γ-carboxyglutamate are not much stronger than those involving aspartate (see above). The explanation is, at least in part, that γ-carboxyglutamate is less flexible than phosphotyrosine and therefore cannot optimize its hydrogen bonds so well.

Finally, salt-bridges involving the 1-form of phosphotyrosine are weaker than those with the 2-form, but the (i, $i + 4$) salt-bridge with arginine is nonetheless stronger than any of the histidine−aspartate salt-bridges examined above. This probably results from the formation of two, rather than one, hydrogen bonds between the two groups.

Discussion

Energetics of Solvent-Exposed Salt-Bridges. This paper analyses the energetics of solvent-exposed ion pairs with a novel computational methodology. The computational method is found to be accurate to within a few kJ/mol, on the basis of comparisons with measured association energies of ions in solution and with the energies of salt-bridges extracted from studies of α-helix stability. Thus, both experiment and computation indicate that the formation of a solvent-exposed salt-bridge between monovalent groups gains roughly 2.0−4.0 kJ/mol. Although such salt-bridges are weak, they can nonetheless be physiologically important. The classic example is hemoglobin, where a large number of weak, solvent-exposed salt-bridges together contribute significantly to cooperativity.[1]

Despite this evidence that solvent-exposed salt-bridges are stabilizing, it has proved difficult to design mutations that reliably introduce stabilizing salt-bridges into T4 lysozyme.[29] This result contrasts with the peptides studied here, where salt-bridges seem to stabilize the helical conformations quite

reliably.[13−16] One reason for the difference may be that the helical peptide studies are often done at ionic strengths of <50 mM, while the mutant study of Dao-Pin et al. was done at about 150 mM ionic strength. The higher salt concentration is expected to weaken electrostatic interactions. However, other studies of helical peptides do show that salt-bridges remain stabilizing even at ionic strengths well above 150 mM,[15,16] so ionic strength is not the full explanation for the difference relative to the T4 lysozyme study.

We suggest that another part of the explanation concerns the choice of reference state, as previously discussed.[24] That is, the state of the system when the salt-bridge is absent is critical in determining the reported energetics of the salt-bridge. For the helical peptide studies considered here, the (i, $i + 4$) salt-bridge is broken by increasing the sequence gap from 4 to 5, *not by deleting the charged groups from the peptide*. In contrast, for the T4 lysozyme mutants, the salt-bridges are "broken" by deleting one or both charged groups. This results in a very different reference state because adding charged groups to certain sites on a protein can be intrinsically destabilizing, as observed by Dao-Pin et al for the addition of histidine and aspartate side chains. As a consequence, replacing neutral side chains by charged ones may destabilize the native state even if the charged side chains form a salt-bridge. A related point is that the neutral side chains that were mutated to charged ones in T4 lysozyme may themselves have had a significant stabilizing interaction, such as hydrophobic interactions or hydrogen bonds. The data in Table 4 indicate that solvent-exposed hydrogen bonds in which one partner is ionized can confer almost as much stability as charge−charge interactions.

The same distinction between reference states holds for salt-bridges in helical peptides; as pointed out by Lyu et al., the neutral forms of lysine and glutamic acid in isolation are more helix-stabilizing than their ionized forms.[16,56−58] This implies that adding charges to a peptide can, by itself, make the peptide less helical. This decrease in helicity can, however, be offset if the charges form salt-bridges. The result is that the net change in the overall stability of the helical state is difficult to predict.

It is clear from this discussion that if one wishes to stabilize a protein by engineering new salt-bridges, it is essential to pay close attention to the environment of the charged groups that are to be added. The mere fact that they are positioned to form a salt-bridge does not guarantee that their addition will stabilize the native state. Clear rules for positioning added salt-bridges to stabilize proteins do not currently exist. However, existing data suggest that it is particularly difficult to stabilize a protein with salt-bridges that are not highly exposed to solvent.[24,17] Indeed, the degree of solvent exposure of the added charged groups in T4 lysozyme correlates well with the degree of stabilization they afford; the three involving a charged group that was >50% buried were destabilizing, and the three in which all charged groups were <50% buried were stabilizing. Further analysis of thermostable proteins, which typically possess more salt-bridges than their mesophilic homologues,[2−9] may offer additional clues on how to design salt-bridges that stabilize proteins. New theoretical studies may also provide new insights into this problem.[59]

These considerations suggest that it may be easier to design salt-bridges to influence allosteric transitions than to stabilize the native state of a protein. This is because allosteric transitions can change the distances between charged groups at domain boundaries without other large conformational changes. Such distance changes may be modeled well by changes in the sequence gap between histidine and aspartate in the helical peptides. One might thus envision adding or deleting charge pairs across a domain interface in order to alter the relative stabilities of two alternative conformations of a protein in a controlled fashion.

The present calculations provide evidence that the charged side chains in helical peptides do not form structurally well-defined salt-bridges. Thus, the analysis of side chain fluctuations in Results shows considerable mobility, with intermittent formation and breakage of salt-bridged conformations. This mobility accounts in part for the small degree of stabilization (∼3.0 kJ/mol) afforded by these side chain interactions, for we compute a larger energy ($U + W$) (∼5.0 kJ/mol) on going from the most stable (i, $i + 5$) conformation to the most stable (i, $i + 4$) conformation, which is salt-bridged. Our result that weakly stabilizing charge−charge interactions can go along with a high degree of side chain mobility is in agreement with the crystallographic study of designed salt-bridges in T4 lysozyme.[29] We also agree that salt-bridges between groups that are held in position by the rest of the protein have the possibility of being stronger than those in which the side chains are free to move about.

Ion-Pairing in Solution. Although the standard free energies of binding for the ion pairs in solution, a few kJ/mol, are rather similar to the salt-bridge energies just discussed, it is worth keeping in mind that there is not a clear fundamental basis for this similarity. This is because standard free energies of binding depend on the standard concentration used in reporting the results: the greater the standard concentration, the more negative the binding free energy. Therefore, it may be of more value to keep in mind the change in $\langle U + W \rangle$ upon binding, $\Delta\langle U + W \rangle$, because this quantity does not vary with the standard concentration. As noted in Results, our calculations indicate this value is about −10.0 kJ/mol for guanidinium and phosphate in solution, in excellent agreement with a previous study[55] that interpreted experimental data with theory due to Williams[60] and Paige and Jencks.[61]

On the other hand, our calculations showing weak association of counterions in solution contrast sharply with a recent computational study[62] indicating that an ion pair in water (methylammonium−methyl acetate) is at least 80.0 kJ/mol less stable than the separated ions; that is, oppositely charged ions were predicted to repel each other strongly in solution. Experimentally, however, counterions, and in particular ammonium and acetate groups, do indeed form weak ion pairs,[35] in agreement with our results and in disagreement with those of No et al.[62] It is possible that the solvent model used in the previous study overestimates the solvation cost of bringing together oppositely charged ions in water.

Because the ion pairs examined here are almost rigid—the only motion is the rotation of the phosphate hydroxyl(s)—the difference between $\Delta\langle U + W \rangle$ and the standard free energy of binding is essentially equal to the change in external (translational and rotational) entropy.[40] (Changes in solvent entropy are implicit in the $\langle U + W \rangle$ part of $\Delta\langle U + W \rangle$.[40]) That is, as previously discussed,[40]

$$\Delta G^\circ_{\text{bind}} = \Delta\langle U + W \rangle - T\Delta S^\circ_{\text{ext}} \tag{7}$$

This equation can be used to estimate $\Delta S^\circ_{\text{ext}}$. Using the value of −10.0 kJ/mol for $\langle U + W \rangle$ and −4.0 kJ/mol for $\Delta G^\circ_{\text{bind}}$ (Table 3) yields $\Delta S^\circ_{\text{ext}} = -20.0$ J mol^{-1} K^{-1}. This value is smaller than previously calculated changes in external entropy for the binding of benzene by T4 lysozyme (97.0 kJ mol^{-1} K^{-1}).[63] That the entropy cost for guanidinium−phosphate binding is lower may result from the fact that the phosphate binds to the

guanidinium in eight distinct, doubly hydrogen-bonded conformations. These multiple bound conformations allow more entropy to be retained upon binding than would a single binding site and orientation.

Strong Salt-Bridges with Phosphotyrosine. The present calculations suggest that salt-bridges involving phosphotyrosine will be about twice as strong as those involving monoanionic side chains. In the context of an (i, $i + 4$) interaction, they also imply that an arginine partner will yield greater stability than a lysine (Table 5). However, it should be emphasized that the difference between lysine and arginine in Table 5 probably has more to do with the steric constraints imposed by the helical conformation than with the intrinsic properties of ammonium and guanidinium groups. This is because both experiment and calculation show that butylammonium and guanidinium interact similarly with phosphate in solution, where the ion pairs are free to adopt their most stable conformations. Thus, the difference in the salt-bridge energies for lysine and arginine with phosphotyrosine might be smaller in a real peptide than in the rigid model peptides used in the present calculations. This is because a real peptide retains some flexibility in the helical form. This flexibility might allow lysine to assume the conformation needed to form a strong salt-bridge with phosphotyrosine. The same might be true for γ-carboxyglutamate, although the more diffuse charge distribution on this side chain may lead in general to salt-bridges weaker than those with phosphate.

It is worth making a definite statement of what the calculations do and do not predict for phosphotyrosine-containing peptides. They predict that a potentially helical peptide will be significantly more helical when it contains arginine and phosphotyrosine in an (i, $i + 4$) relationship, than when it contains arginine and phosphotyrosine in an (i, $i + 5$) relationship. However, the calculations do not bear directly on the relative helicity of a peptide with arginine and tyrosine in an (i, $i + 4$) relationship compared to the helicity of the same peptide after the tyrosine has been phosphorylated. This is because we are unable to predict the intrinsic effect of phosphorylation itself upon helix stability. The distinction here is the same as that made above in the discussion of references states for salt-bridges. On the other hand, some salt-bridges do indeed stabilize native states (see ref 15 and references therein), so an arginine−phosphotyrosine salt-bridge might produce a net increase in stability. Thus, it might be possible to synthesize peptides with several arginine and tyrosine residues in (i, $i + 4$) relationships that would become helical when incubated with tyrosine kinase and ATP and would revert to coil when exposed to phosphatase. A spectroscopic probe linked to such a peptide could serve as a real-time indicator of relative levels of kinase and phosphatase activity.

Acknowledgment. We thank B. Tidor for his thoughtful comments on the manuscript. Certain commercial equipment or materials are identified in this paper in order to specify the methods adequately. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Abbreviations

CD	circular dichroism
FDPB	finite difference Poisson−Boltzmann
GB	generalized Born
HIV	human immunodeficiency virus
MM	mining minima
PB	Poisson−Boltzmann
TMA	tetramethylammonium

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