THE STABILIZATION ENERGY OF THE GLU–LYS SALT BRIDGE IN THE PROTEIN/WATER ENVIRONMENT: CORRELATED QUANTUM CHEMICAL *ab initio*, DFT AND EMPIRICAL POTENTIAL STUDIES

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Dedicated to Professor Rudolf Zahradník on the occasion of his 80th birthday.

The stabilization energies of Glu–Lys salt bridges are calculated at the CCSD(T) complete basis set limit to provide a reasonable description of the strength of the ion-pair bond in the gas phase. The effect of the environment (protein with ε = 4 and water with ε = 80) on the stabilization energy was introduced via a modification of the quantum chemical DFT energy, for which the COSMO methodology was adopted. The other (standard) approach was based on incorporating a dielectric constant into the Coulomb electrostatic term of the Amber empirical potential function and utilizing the generalized Born model implemented in the Amber program. The environment affects the stabilization energy of the salt bridge dramatically: The protein reduces the energy to less than one half of the original value, whereas water sometimes changes stabilization to destabilization. Both theoretical procedures, based on completely different theoretical backgrounds, yield very similar results, which strongly support their validity. An ion pair is converted to an ion-neutral pair when its pH is changed. This transformation is connected with a strong reduction of the stabilization energy regardless of the environment. The substantial differences in the stabilization energies of ion pairs and ion-neutral pairs contradict the negligible changes of free energy detected experimentally. Evidently, the contribution of formation and hydration entropy is significant and compensates for the large stabilization energies.

Keywords: Salt bridges; Ion pairs; Protein stabilization; Interaction energies; Protein dielectric; *Ab initio* nonempirical calculations; Complete basis set.

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Non-covalent interactions, along with cystein bridges, determine and stabilize the structure of proteins[1]. They can be classified according to their origin or strength into the following classes: hydrogen (H)-bonding, electrostatic, induction (which covers charge-transfer energy), van der Waals, and hydrophobic interactions[2]. Hydrogen bonds are abundant in proteins, be they intramolecular or with solvent molecules, and are commonly believed to be the most important forces for protein structure and stability. Depending on the participating amino acids, various X–H···Y hydrogen-bonded motifs exist. The largest stabilization is found for X, Y = O, N, C and S, but non-negligible contributions also originate from X–H···π interactions, mainly because of their abundance[3]. H-bonds consist of electrostatic, induction and dispersion contributions. Almost pure electrostatic interactions, which originate from the presence of two charged subsystems, are also present in proteins with the best known example being a salt bridge, which is an ion pair of two charged amino acid side chains. The geometrical definition of a salt bridge from 1983 [4,5] requires a distance of 4.0 Å between the charged groups of centroids and the existence of at least one pair of side-chain nitrogen and oxygen atoms within a 4.0 Å distance. This electrostatic interaction element seems to be a key factor in molecular recognition, protein–protein interaction, flexibility and thermostability[6]. It can also play a very important role in the structure and stability of proteins. The strength of the electrostatic attraction between positively and negatively charged subsystems is substantial (by an order of magnitude larger than other contributions) and nearly approaches the strength of a covalent bond. This is true, however, only in the gas phase or in salt crystals. In any other medium, dielectric screening reduces the magnitude of the charge distribution and thus the strength of the electrostatic term. An extreme case is represented by the water phase, where the electrostatic interaction is reduced dramatically by the hydration of both charged partners. This is the case of a salt bridge located at the protein surface, which is thus directly exposed to the water phase. If a salt bridge is partially buried in the protein interior, the situation might be quite different and there is no unambiguous opinion about its strength. Some analyses have shown that the electrostatic term is negligible in this case[7] while others have indicated significant stabilization[8,9]. This point is of key importance in the case of thermophilic proteins as the thermostability of hyperthermophilic proteins may be related to the abundance of salt bridges[10,11].

The uncertainty about the significance of electrostatic interactions in a medium is understandable in light of the difficulties connected with this study[11]. The experimental findings[12] are surprising: Upon mutation of one

of the two Ser–Lys pairs to Glu–Lys salt bridges, a rather small stabilization of about 1.2 kJ/mol resulted. When both pairs were mutated simultaneously, only a slightly larger stabilization of 3.6 kJ/mol was obtained. Without doubt, these values are small, and it is evident that the stabilizing and destabilizing contributions nearly cancel each other. However, it should be emphasized that the experiments determine the changes of the total free energy in an environment. This value can be approximately expressed (see also Kumar and Nussinov[7]) as a sum of the ion-pair interaction energy (enthalpy), the pair formation entropy, the hydration (dehydration) free energy of the pair, and the interaction free energy of the pair with the rest of the protein. Among these terms, the ion pair stabilization energy seems to be the largest. It should be kept in mind, however, that it does not correspond to the gas-phase stabilization energy of the pair, because the pair is surrounded by an environment, which affects its electron distribution, and, therefore, also its stabilization energy. The decomposition of the total value into its components is desirable, since it helps understand the nature of the process, but the experimental decomposition is very complicated, if not impractical. The theoretical approach, on the other hand, is well suited for this purpose, but each term has to be evaluated accurately to avoid (at least some) error compensation.

It is difficult to cover the role of the environment adequately and accurately in a theoretical study. The classical approach, which is based on a reduction of the electrostatic contribution through the dielectric constant, is used in many empirical potentials, and several modifications have been suggested[13]. It is worth mentioning also the work of Warshel[14,15], which reviews dielectric constants in proteins, here and Wade's[16] study of exceptionally stable salt bridges in cytochrome P450cam. The role of the environment is incorporated into Coulomb's law through the dielectric constant ε. These models are valid at large distances but questionable when the ions are in close contact. In this work, we have included them to examine their performances for such cases. If ε is actually a variable of the respective interatomic distance, the so-called "distance-dependent dielectric constant" approach has been adopted. Even though the introduction of a dielectric constant is a physically correct modification of the Coulomb energy term, it does not affect all the other electrostatic-based terms, such as the induction energy. Evidently, a physically justified model of screening by a dielectric medium requires the correction of all electrostatic terms. This can be achieved by a modification of the electronic energy in a quantum chemical calculation. There are many continuum solvent-based methods, which have so far been used mainly for the estimation of hydration free energies.

Nevertheless, the same methodology is able to yield the electronic interaction energy affected by any continuous solvent.

The aim of this paper is to investigate the strength of various Glu–Lys ion pairs in the protein rubredoxin as well as their ion-neutral counterparts in which either the Glu is protonated or the Lys is deprotonated. It is known that the salt bridge of the side chains between Lys6 and Glu49 does not stabilize the hyperthermophilic rubredoxin (Rd) variant[9]. In addition to this salt bridge in a wild type and a mutant form of rubredoxin from *Pyrrococus furiosus* (Pf) Rd, we have also explored the Glu–Lys interaction localized between residues that are partially buried inside the protein interior. They differ in terms of the distance between the two ionic heads of the side chains. In all these cases, we considered the dependence of the electronic energy on the dielectric constant of the protein environment as well as of the solvent. For the first time, we have utilized the concept of total electronic energy and its variant in a continuous protein environment. The knowledge of the total electronic energy, modified by the environment, allows for the construction of the total interaction energy affected by the environment, including the various interaction energy terms like Coulomb, induction and charge transfer, which also covers the effect of the basis sets superposition.

METHODS

Systems Studied

As stated above, there is good reason to study proteins from a thermophilic organism or their mesophilic counterparts to address the origin of their thermostability. As a system for our study, we have chosen the small protein rubredoxin, of which various ion pairs between the Glu and Lys side chains were selected. The coordinates of all the interacting pairs were obtained from crystal structures of the hyperthermophilic rubredoxin from *Pyrrococus furiosus* (pdb code: 1BRF) or its mutants (pdb codes: 1BQ9, 1IU5) and its mesophilic counterpart (pdb code: 1SMM). The amino acids forming salt bridges were excised from the protein and their N termini were set to NH_2 and O termini to H–C=O, i.e. not in a zwitterionic form. It has been shown by Strop and Mayo[9] that there is a side chain to the side-chain salt bridge between the Lys6 and Glu49 in hyperthermophilic rubredoxin Pf Rd, which was found not to stabilize the protein. In addition to this pair in the wild type of Pf Rd (1BRF – annotated as **SB6**), we also studied the Glu47–Lys6 pair in a mutant (W3Y, I23V, L32I) of the Pf Rd (1IU5 – marked

as **SB1**), the Glu49–Lys6 pair in the formyl-methionine mutant of Pf Rd (1BQ9 – labeled as **SB2**), and the Glu54–Lys2 pair in *Clostridium pasteurianum* (Cp) Rd (1SMM – labeled as **SB3**). We also studied two further salt bridges in Pf Rd (1BRF), the Lys50–Glu30 (marked as **SB4**) and Lys50–Glu52 (labeled as **SB5**) ion pairs. This set of ion pairs provides a variety of spatial arrangements of the charged termini, some of which are located on the surface while others are partially buried inside the protein structure (Fig. 1). All the amino acid pairs considered are depicted in Fig. 2. The distance between their charged termini ranges from 3.27 to 5.70 Å. The positions of all the hydrogen atoms, which have not been determined by X-ray methods, were optimized at the B3-LYP/6-31G** level.

Strategy of Calculations

Interaction energies of various types of intermolecular clusters (ionic, H-bonded, stacked, etc.) depend differently on the detailed quality of the calculation: the size of the AO basis set and the level of correlation energy. The only comparable level is a calculation with an infinitive AO basis set covering a substantial portion of the correlation energy. Hence, the inter-

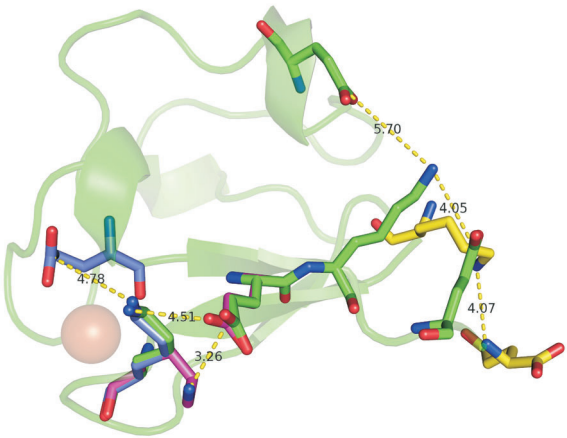

FIG. 1
Structure of wild-type rubredoxin from *Pyrrococus furiosus* (Pf Rd) with salt bridges involved in this study. All other rubredoxin structures (1IU5, 1BQ9, 1SMM) were aligned to the structure of wild-type (1BRF, green). Salt bridges differ in color (**SB1**, blue; **SB2**, violet; **SB3**, yellow) from those of the wild-type (**SB4**–**SB6**, green). The distance (in Å) between the COO^- carbonyl carbon and NH_3^+ nitrogen is shown for each salt bridge

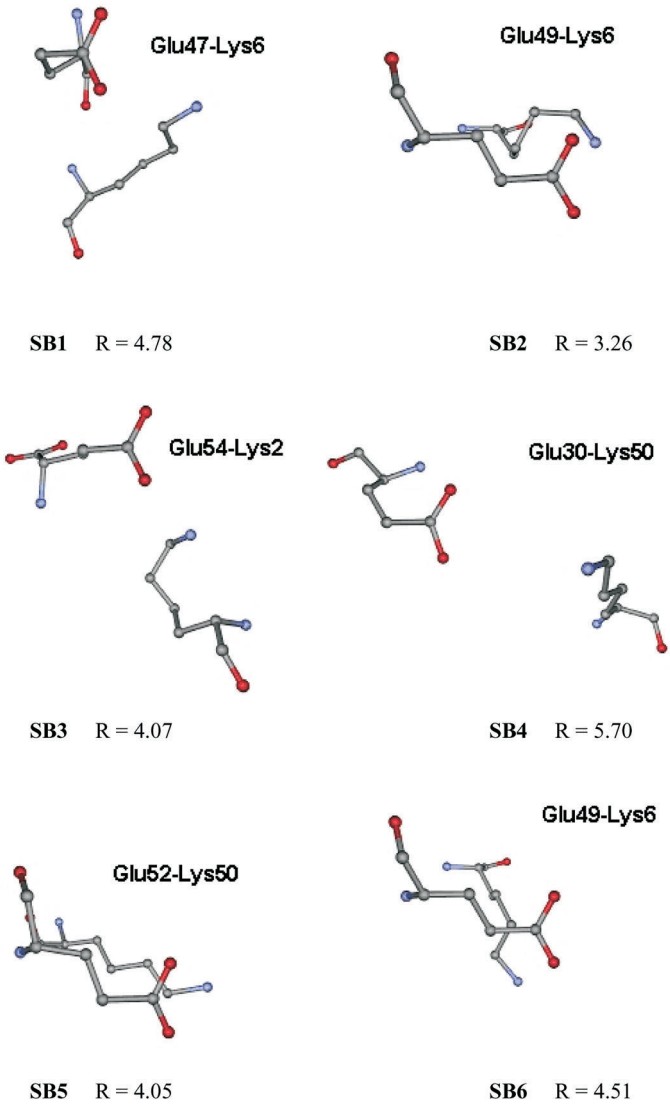

FIG. 2
A perspective view of the Glu–Lys ion pairs investigated; R is the distance (in Å) between the COO^- carbonyl carbon and NH_3^+ nitrogen. The carboxylate plane is used as a reference for the superposition

action energy of the present complexes has been approximated by a complete basis set (CBS) limit of the CCSD(T) interaction energy. The CCSD(T) interaction energies were shown to be close to accurate values[17] and the CCSD(T) CBS interaction energies of the DNA base pairs agreed well with the experimental results[18].

The CCSD(T) CBS interaction energies were determined for the Glu–Lys salt bridges using experimental geometries taken from X-ray structures with the positions of all the hydrogens optimized by the DFT (B3LYP/6-31G**).

The role of the environment was modeled by the COSMO methodology, which is only implemented at the DFT level. Therefore, the performance of the DFT was first tested for the amino acid pairs in vacuo, where the calculations performed at the CCSD(T) CBS level served as a benchmark. The COSMO interaction energy was determined based on the knowledge of the total DFT electronic energy calculated in a dielectric.

Accurate Interaction Energies

The CCSD(T) interaction energy is approximated as

$$\Delta E^{\mathrm{CCSD(T)}}{}_{\mathrm{CBS}} = \Delta E^{\mathrm{MP2}}{}_{\mathrm{CBS}} + (\Delta E^{\mathrm{CCSD(T)}} - \Delta E^{\mathrm{MP2}})|_{\mathrm{medium\ basis\ set}}$$

where the first and second terms represent the CBS limit of the MP2 interaction energy and the CCSD(T) correction term.

The MP2 stabilization energy was extrapolated to the CBS limit using the two-point scheme of Helgaker et al.[19]. Because of the different convergences of the HF and MP2 energies, both energies were extrapolated to their CBS limits separately on the basis of the cc-pVDZ and cc-pVTZ energies.

The CCSD(T) correction term was calculated with a medium basis set (6-31G** (0.25, 0.15)). This could be done because the CCSD(T)-MP2 interaction energy difference depends much less on the basis set than the MP2 and CCSD(T) interaction energies themselves[20]. Our recent calculations on uracil dimer fully confirmed this conclusion and CCSD(T) correction term determined with 6-31G**(0.25, 0.15) and aug-cc-pVDZ differs only slightly[21].

DFT Interaction Energies

All the DFT calculations were carried out using the TPSS meta-generalized gradient functional[22] and the TZVP ([5s,3p,1d/3s,1p]) basis set. TPSS is a pure DFT functional, for which the resolution of identity (RI) approximation[23] was used to obtain a reasonable computational efficiency. The inter-

action energy was obtained as the difference of the energy of the complex and the energies of the monomers. The triple-zeta basis set is large enough to keep the BSSE small (~1 kcal/mol), and so no counterpoise corrections were made. The dispersion energy, which is not covered by the DFT methods, was included as an empirical correction[24].

Dependence of the DFT Electronic Energy on the Dielectric Constant

The conductor-like screening model (COSMO)[25] was used to introduce a polarizable protein or solvent environment into the DFT calculations. Despite its not being directly intended for this purpose, the model can be used for the calculation of the interaction energy of a complex in a dielectric environment. We have not attempted to calculate the stabilization Gibbs energy, which describes the separation of the interacting residues. Since we were interested in how the environment affects the interaction in a given geometry, only the electrostatic contribution to the solvation Gibbs free energy had to be calculated in this analysis. An important feature is the comparable description of the solvent, which must be consistent in all three calculations (supersystem R...T, subsystem R, subsystem T) to allow for a subtraction of the subsystems' energies from the energy of the supersystem. For the calculation of the energy of a single amino acid, the other amino acid was represented by dummy atoms with the same atomic radii as the real atoms. Identical cavities were thus generated for the calculations of the monomers and the complex. We are aware that the value of the dielectric constant is a very important parameter of the calculation and differs depending on the systems and authors[14,15]. We decided to model three different constants and in some cases extreme types of the environment. A dielectric constant of $\varepsilon = 80$ was used to model the water environment when the residue was entirely exposed to the solvent, whereas $\varepsilon = 4$ was chosen as an average dielectric constant of the protein interior, e.g. a residue completely buried inside a protein. Finally, the dielectric constant equal to 1 was adopted for modeling the gas phase.

Electrostatic Energy and the Effect of the Dielectricum

For method comparison, all the interaction energies were also calculated using molecular mechanics. The Cornell et al. force field[13] was employed as implemented in the Amber 8.0 package[26]. When using molecular mechanics, the interaction energy consists of the van der Waals and the electrostatic terms only. The polarizability of the environment was introduced via

the dielectric constant used for all electrostatic interactions, calculated using Coulomb's law. The resulting interaction energies were compared with the pair-wise stabilization energies computed with generalized Born solvation model (GB).

Software Used

All RI-MP2 and RI-DFT calculations were done using the Turbomole package[27]. The CCSD(T) correction term was calculated with the Molpro 2006 [28]. Amber 8.0 package[26] was used for calculation of molecular mechanic interaction energies and pair-wise stabilization energies with GB solvation model.

RESULTS AND DISCUSSION

Table I shows the partial and CBS limit MP2 interaction energies of the six salt bridges studied. Following our expectations, all the stabilization energies are very large, larger than 60 kcal/mol, and in one case even larger than 100 kcal/mol. This is in agreement with the results of Aleman et al.[29], who calculated the interaction energies of ion pairs in the gas phase. The interaction energies depend only slightly on the AO basis set size and the CBS limits are close to both the cc-pVDZ and cc-pVTZ interaction energies. The CCSD(T) correction terms are systematically negligible, and the total interaction energies are thus practically identical with the MP2 CBS energies.

TABLE I
The MP2 and CCSD(T) interaction energies (in kcal/mol) for Glu–Lys salt bridges

System	ΔE^{MP2}			ΔCCSD(T)	ΔE
	cc-p-VDZ	cc-p-VTZ	CBS		
SB1	–80.21	–80.61	–80.78	0.05	–80.73
SB2	–115.39	–114.11	–113.57	0.22	–113.35
SB3	–109.83	–94.52	–88.06	–0.23	–88.29
SB4	–58.47	–59.71	–60.23	–0.13	–60.36
SB5	–99.35	–97.78	–97.12	–0.02	–97.14
SB6	–72.48	–73.72	–74.24	–	–

[a] See Fig. 2.

Table II shows the DFT/TPSS/TZVP interaction energies and the contribution of empirical dispersion energies. As expected, their sum is close to the above-mentioned data, which we have used as benchmark values. The absolute average error is roughly 3 kcal/mol. The dispersion contribution to the interaction energy remains constant when the environment is changed. Therefore, the DFT/TPSS/TZVP interaction energy itself can be used for further studies of the role of the protein environment.

Having compared the DFT interaction energies determined in the gas-phase and in the protein environment (columns 3 and 4 of Table II), we found a dramatic reduction when passing from the gas phase to the protein environment. The resulting ratios lie between 0.23 and 0.43, i.e. the protein reduces the stabilization energy of a salt bridge to less than 43% of the stabilization in the gas phase. It needs to be emphasized here again that the distances between the charged heads of both amino acids vary from 3.3 to 5.7 Å, and also the arrangement of the two ion side chains is different. In agreement with our expectations, the dependence between the reciprocal distance and the reduction factor is approximately linear.

When passing from the gas-phase environment to water, where $\varepsilon \approx 80$, the stabilization decreases dramatically (column 5 in Table II). In four out of the six cases studied, the introduction of a water environment changed stabilization to destabilization, and only for the two pairs with the shortest

TABLE II
The dispersion energies and the DFT/TPSS/TZVP interaction energies (in kcal/mol) in vacuo, a protein environment ($\varepsilon = 4$) and a water environment ($\varepsilon = 80$) as well as their ratios for the Glu–Lys salt bridge in the different environments

System[a]	ΔE^{DFT}				$\Delta E^{DFT}(\varepsilon=4)/\Delta E^{DFT}(\varepsilon=1)$	$\Delta E^{DFT}(\varepsilon=80)/\Delta E^{DFT}(\varepsilon=1)$
	E^{Disp}	$\varepsilon = 1$	$\varepsilon = 4$	$\varepsilon = 80$		
SB1	–4.28	–73.92	–24.66	5.52	0.33	–0.075
SB2	–4.67	–105.45	–43.36	–4.64	0.41	0.011
SB3	–1.80	–91.77	–36.98	–1.26	0.40	0.013
SB4	–0.26	–60.76	–20.79	2.02	0.34	–0.033
SB5	–4.90	–89.02	–33.20	1.62	0.37	–0.018
SB6	–4.20	–65.96	–15.33	8.07	0.23	–0.012

[a] See Fig. 2.

distance between the charged heads did the contribution remain slightly stabilizing. This finding is physically correct, because when the distance between polar heads is short, the water environment affects the interaction only from the outside. For larger distances, water additionally penetrates the charged heads, and this effect is decisive for changing the stabilization effect to destabilization.

The real value of salt bridge stabilization in a protein is somewhere between the results obtained for the protein environment and for water. The salt bridge considered can be partially buried inside the protein or more exposed to the solvent, and it is very important to take this detail into account despite the difficulty of its quantification.

The gas-phase interaction energies obtained by molecular mechanics (MM, Table III, column 3) are in good agreement with the benchmark data. Electrostatics dominate the interaction, with the contribution of the van der Waals interactions (dispersion and repulsion, column 2) being small. In protein (ε = 4), the electrostatic term is reduced to exactly 25%, and the molecular mechanics interaction energy is reduced to about 27%. As compared to the DFT results, the destabilizing effect of the environment is slightly stronger, but both values basically agree. Keeping in mind that the role of the environment is described in both procedures entirely differently, the reasonable agreement between the two values is a strong argument in

TABLE III
The MM interaction energies and their van der Waals contributions (in kcal/mol) of the Glu–Lys salt bridges in vacuo, a protein environment (ε = 4) and a water environment (ε = 80) as well as their ratio for the Glu–Lys salt bridge in the different environments

System[a]	ΔE^{MM}				$\Delta E^{MM}(\varepsilon=4)/\Delta E^{MM}(\varepsilon=1)$	$\Delta E^{MM}(\varepsilon=80)/\Delta E^{MM}(\varepsilon=1)$
	E^{vDW}	ε = 1	ε = 4	ε = 80		
SB1	–2.66	–73.79	–20.44	–3.56	0.28	0.048
SB2	–3.16	–101.4	–27.72	–4.41	0.27	0.043
SB3	–1.75	–87.53	–23.2	–2.84	0.27	0.032
SB4	–0.34	–60.56	–15.4	–1.11	0.25	0.018
SB5	–4.29	–87.74	–25.15	–5.35	0.29	0.06
SB6	24.46	–41.85	7.89	23.62	–0.18	–0.5

[a] See Fig. 2.

favor of the reliability of the two procedures. The interaction energies between the charged pairs in water environment (ε = 80) again show a significant drop from the gas-phase or protein environment values. The difference between the MM and DFT interaction energies in water is rather large, in the case of the **SB1** and **SB5** pairs greater than 7 kcal, and the DFT values are without doubt more reliable. The **SB6** ion pair is an exception, with the shortest hydrogen–hydrogen distance being 1.7 Å, and the mutual hydrogen–hydrogen repulsion is large. Consequently, also the van der Waals contribution is large and repulsive (cf. Table III, column 1). This is quite surprising because of the quality of the X-ray structure being high (a resolution of 0.95 Å). The only explanation for such a strong repulsion would be an effect of the repulsion term in MM, which may be overestimated.

It should also be also mentioned that the environment dramatically changes the electrostatic contribution of the stabilization but leaves the dispersion term nearly unchanged. The electrostatic contributions are dominant, and the total sum of all interactions depends greatly on the environment.

Table IV shows the interaction energies in environment computed via the GB solvation model and also with the scaling of the electrostatic term by the dielectric constant. The values differs slightly ($rmsd_{\varepsilon=4}$ = 1.13 and $rmsd_{\varepsilon=80}$ = 1.49 kcal/mol), but it has not escaped our notice that the trend

TABLE IV
The comparison between interaction energy (in kcal/mol) calculated by GB solvation model with values calculated with scaling of electrostatics term

System[a]	ΔE^{GB}		ΔE^{MM}	
	ε = 4	ε = 80	ε = 4	ε = 80
SB1	–17.17	0.74	–20.44	–3.56
SB2	–28.45	–5.37	–27.72	–4.41
SB3	–23.29	–2.96	–23.2	–2.84
SB4	–14.84	–0.38	–15.4	–1.11
SB5	–22.90	–2.39	–25.15	–5.35
SB6	10.08	26.51	7.89	23.62

[a] See Fig. 2.

of the environment effect is similar and agrees well in both computation methods.

The existence of a salt bridge strongly depends on pH. Through varying the pH value, one of the two subsystems eventually becomes neutral, as a result of which the strong ion-pair electrostatic attraction is lost, with a weaker ion-dipole electrostatic attraction occurring instead. Table V shows the interaction energies of the neutral-cation Glu–Lys pair in vacuo, in a protein (ε = 4) and in water (ε = 80) as determined by the DFT and MM methods. The results of similar calculations for the anion-neutral Glu–Lys pair are shown in Table VI. While the parent ion–ion pairs are strongly attractive, the neutral-ion pairs in vacuo are considerably less attractive and the neutral-ion **SB1**, **SB4**, and **SB6** pairs are even destabilizing. This trend is stronger when passing from the gas phase to protein/water environment. It is again important that both procedures provided very similar results and basically agreed excellently in describing the effect of the environment.

Comparing the stabilization energies of the salt bridges and parent ion-neutral pairs, we conclude that they both differ dramatically regardless of the environment. This physically quite reasonable conclusion is in deep disagreement with the experimental findings showing very similar stabilization of salt bridges and neutral-ion pairs. Specifically, the experimentally detected changes of the total free energy of ion pairs and ion-neutral pairs are practically identical[12], whereas the present stabilization energies of both

TABLE V
The interaction energies (in kcal/mol) of neutral-cation pairs in vacuo, a protein environment (ε = 4) and a water environment (ε = 80)

System[a]	ΔE^{MM}			ΔE^{DFT}	
	ε = 1	ε = 4	ε = 80	ε = 1	ε = 4
SB1	–1.79	–2.49	–2.71	1.44	6.20
SB2	–10.07	–4.95	–3.33	–9.81	–0.6
SB3	–12.68	–4.5	–1.91	–14.62	–4.01
SB4	2.81	0.43	–0.32	1.82	2.70
SB5	–6.0	–4.72	–4.31	–2.92	3.38
SB6	29.2	30.59	31.03	6.0	9.48

[a] See Fig. 2.

pairs differ dramatically. This significant difference clearly indicates the decisive role of entropy (assuming that both the interacting side chains are in a similar arrangement), with contributions from both formation and hydration entropy. To compensate for the significantly different stabilization energies, the entropy contributions must be large in the case of ion pairs and much smaller in the case of ion-neutral pairs.

TABLE VI
The interaction energies (in kcal/mol) of anion-neutral Glu–Lys pairs in vacuo, a protein environment (ε = 4) and a water environment (ε = 80)

System[a]	ΔE^{MM}			ΔE^{DFT}	
	ε = 1	ε = 4	ε = 80	ε = 1	ε = 4
SB1	–5.95	–3.41	–2.61	–6.92	1.17
SB2	–13.36	–5.75	–3.34	–9.24	–1.30
SB3	–14.39	–4.91	–1.91	–13.00	–3.96
SB4	–5.38	–1.6	–0.41	–4.64	–0.32
SB5	–8.97	–5.47	–4.36	–8.98	–0.34
SB6	18.31	23.45	25.08	–2.01	5.17

[a] See Fig. 2.

We must add here that we do not claim that our model is a benchmark regarding the approximation of the environment by a dielectric constant of ε = 4 and 80. Rather, we have mapped the range delimited by these two extreme values. We are aware that the approximation of uniform dielectric constant is severe and that we have disregarded the structure and dielectric effects of the individual water hydration networks. Taking into account the individual water structures could conceivably change some of the stabilizing/destabilizing interaction proportions but does not affect the trend obtained by our calculations.

CONCLUSIONS

1. The CCSD(T) CBS stabilization energies of the Glu-Lys salt bridges determined for the experimental geometries are very large, reaching, and in one case even exceeding, 100 kcal/mol. These values represent new benchmark data for this type of ion-pair amino acids in the gas phase.

2. The DFT/TPSS/TZVP interaction energies are close to the benchmark data, especially if empirical dispersion energies are included. The dispersion energies themselves are, however, rather small.

3. The effect of the environment on the electronic energy is of key importance. The protein environment (ε = 4) reduces the stabilization energy of salt bridges by 23–43% and an even larger reduction occurs when the water environment is considered, which sometimes changes large stabilization to destabilization.

4. The strong stabilization of the Glu–Lys salt bridge is lost upon protonation/deprotonation to an ion-neutral amino acid pair as a consequence of the altered pH. This effect is independent of the environment. The large difference between the stabilization energies of the ion pairs and ion-neutral pairs as well as the small difference between the corresponding free energies indicate the decisive role of entropy, which should be large for the former pairs and small for the latter pairs.

5. It is obvious that the **SB6** pair between Glu49–Lys6 in hyperthermophilic rubredoxin 1BRF is in an unfavorable arrangement, and its role as a salt bridge is questionable. On the other hand, the same ion pair, localized in the structure of a mutant of Pf Rd, is in a favorable orientation and shows significant stabilization in all the environments. We can conclude that not all ion pairs can contribute to the overall stabilization and that this fact strongly depends on the spatial arrangement and distance between the ionic heads.

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