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Theoretical study of arginine–carboxylate interactions

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Abstract

The importance of the guanidinium–carboxylate interactions has sprung from the observed salt bridges often present in biological systems involving the arginine–glutamate or arginine–aspartate side chains. The strength of these interactions has been explained on the basis of a great coulombic energy gain, due to the closeness of two charges of opposite sign and the occurrence of H-bond interactions. However, in some environments proton transfer, from guanidinium to carboxylate, can occur with the consequent annihilation of charge. In this work, both ab-initio (6-31G** and MP2/6-31G**) and semi-empirical (AM1) calculations were performed in vacuo on appropriate models, methylguanidinium–acetate and methylguanidine–acetic acid to simulate the zwitterionic and the neutral forms, respectively. The results obtained indicate that, in solvent-free hydrophobic environments, the neutral form should be more stable than the zwitterionic one.

Keywords: Arginine–carboxylate interactions; Conformational analysis; 6-31G**; MP2; Proton transfer

1. Introduction

It is now currently accepted that the three-dimensional structure of proteins is directly related to their biological activity. Therefore, while the amino acid sequence of a particular protein has all the information needed to trigger the biological response, the protein conformation ensures an effective interaction within itself as well as with specific receptor sites. In reality, the interactions between terminal side chains of amino acids in proteins seem to be a determinant factor in the mechanisms of a wide variety of biological phenomena as, for example, the antigen/antibody recognition and enzyme–substrate interactions [1–5]. In particular, those involving ionic groups of opposite charge are expected to be more important because it is generally assumed that their electrostatic contributions to the overall stabilization energy are essential. One special case of this type of interactions involves the guanidinium group of the arginine, which usually defines the binding site of a wide variety of enzymes whose substracts contain carboxyl or phosphate groups [6] and have been the subject of an extensive list of studies [7]. In fact, the occurrence of close interactions with the terminal group of the side chain of arginine is noteworthy, and about 40% of the pairs of ionic groups within

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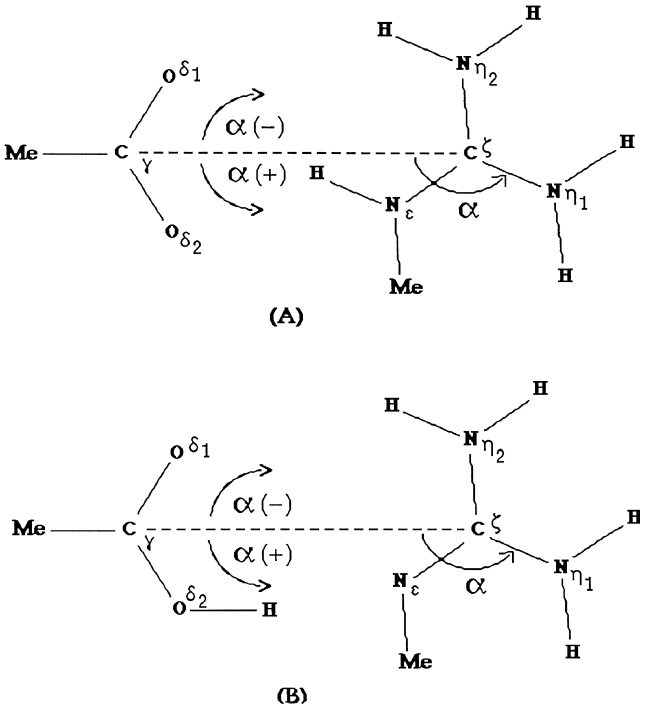

Fig. 1. Schematic representation of methylguanidinium-acetate (A) and methylguanidine-acetic acid (B). In both systems the angle of rotation α, studied in the conformational search, is shown.

proteins involve guanidinium–carboxylate salt bridges and are now quite well documented [7, 8].

So far, the arginine–glutamate (ARG–GLU) and arginine–aspartate (ARG–ASP) interactions, equally of the type guanidinium–carboxylate, have always been associated both by experimentalists [8–14] and theoreticians [14–18] with a zwitterionic state as opposed to a neutral one. However, some recent theoretical studies [19,20] have suggested that, in some environments, the neutral form should be more stable. In fact, using the acetate ion or acetic acid and methylguanidinium ion or methylguanidine as models for the terminal side chain of the glutamate/aspartate and arginine, respectively (see Fig. 1), Melo and Ramos [19] showed that the neutral form is favoured for almost all coplanar conformations in vacuo. Exceptions were observed only for the interactions in which the methyl groups of the guanidinium point towards the carboxylate. A similar result was also obtained by Théry et al. [20] in the peptide model GLY–ARG–GLU–GLY using their 'local-self-consistent-field' (LSCF) method. In this work, we present a more complete quantum mechanical study of the arginine–carboxylate interaction and a systematic discussion of its nature.

Proton transfer, with its consequent charge displacement, and which we have found to occur in the above mentioned interactions, is known as having a key role in many biological mechanisms such as enzymatic reactions and drug-receptor interactions [21–23]. The properties of functional proteins depend on their three dimensional structures which, in turn, depend on the state of the charged side chains of amino acids. These proton transfers between acidic and basic side chains can produce conformational changes in proteins and, therefore, influence their functions [24,25]. Details on the proton transfer mechanism in general have been revealed by theoretical studies and it has been observed that the ab initio calculated potential energy curve is basis set dependent [21,22,25–30]. Therefore, care must be taken in the choice of the basis set of atomic functions employed. Inclusion of polarization functions in the basis set, especially a set of p functions in the

hydrogen atoms, seems more suitable for an accurate description of the proton transfer. In our studies, the 6-31G** basis [31] set has been used as a compromise between the accuracy of the results and the time of computation. The inclusion of the electronic correlation can be also very important for a good description of the potential energy surfaces in proton transfer reactions [26,28–30]. In this work, to evaluate the importance of this effect in the study of arginine–carboxylate interactions, MP2/6-31G** [32,33] calculations have been performed in the most important conformational regions.

The time of computation has always limited the application of accurate ab initio calculations to small model systems, thus preventing the study of larger biological aggregates. In an attempt to be able to study bigger systems with the possibility of solvent effects and, at the same time, ensure a reasonable accuracy in quantum mechanical results, semi-empirical methods were also tested. The AM1 method [34] seems to be the best choice at the semi-empirical level because it was a method specially devised to be applied in systems where hydrogen bonds play an important role. In this work, AM1 calculations have been carried out to evaluate the lower quantum mechanical level which can, on one hand, save computation time and enable the study of bigger systems and, on the other hand, be good enough to describe the arginine–carboxylate interactions.

In total, in this work, we present a quantum mechanical study (6-31G**, MP2/6-31G**, AM1) of a very important interaction systematically occurring in proteins and involved in a series of biological phenomena, namely the arginine–carboxylate interaction; also presented here is a study of the importance that proton transfer has on this same interaction and when it occurs. As mentioned above, the semi-empirical method AM1 was tested and its performance compared to its ab initio counterparts, in an attempt to find out if the method is good enough to handle this type of situations.

2. The nature of the arginine–carboxylate interaction

The interaction between arginine and carboxylate groups is amongst the most important ones occurring between terminal side chains in proteins. The strength of this interaction has been explained on the basis of the ion pair nature of the two inter-residue hydrogen bonds, which are much stronger than those between neutral moieties [8–18]. However, some recent theoretical studies [19,20] have suggested that, in some environments, an inversion to the general tendency could occur with a preferential stabilization of the neutral form. However, no systematic explanation of the nature of the arginine–carboxylate interaction has been presented so far.

Using the supermolecule approach and the above mentioned molecular models (see Fig. 1), the stabilization energies of the zwitterionic ($\Delta E_{\text{zwitt.}}$) and neutral ($\Delta E_{\text{neut.}}$) forms can be calculated according to the following equations,

$$\Delta E_{\text{zwitt.}} = E[\text{MGH}^+ : \text{Ac}^-] - E[\text{MGH}^+] - E[\text{Ac}^-] \quad (1)$$

and

$$\Delta E_{\text{neut.}} = E[\text{MG} : \text{HAc}] - E[\text{MGH}^+] - E[\text{Ac}^-] \quad (2)$$

where $E[\text{MGH}^+{:}\text{Ac}^-]$ and $E[\text{MG:HAc}]$ are the energies of the methylguanidinium–acetate and methylguanidine–acetic acid dimers respectively. In both previous equations, the non-interacting methylguanidinium [MGH^+] and acetate [Ac^-] ions have been considered as the initial reference state. Therefore, the relative stabilization energy ($\Delta\Delta E$) between the zwitterionic and neutral forms can be calculated as the respective energy difference:

$$\Delta\Delta E = \Delta E_{\text{zwitt.}} - \Delta E_{\text{neut.}} = E[\text{MGH}^+ : \text{Ac}^-] - E[\text{MG} : \text{HAc}] \quad (3)$$

In vacuo, this quantity is the result of two opposite effects. The first one is the relative stability of the charged monomers (MGH^+,Ac^-) and the neutral monomers (MG,HAc). This is associated with the different intrinsic proton affinities of an oxygen atom of the carboxylate group and a nitrogen atom of the guanidinium group. Theoretical calculations performed at the 6-31G level [15,16] indicate that this effect favours the neutral form.

The second effect is the different magnitude of the interactions between the charged pair (MGH^+,Ac^-)

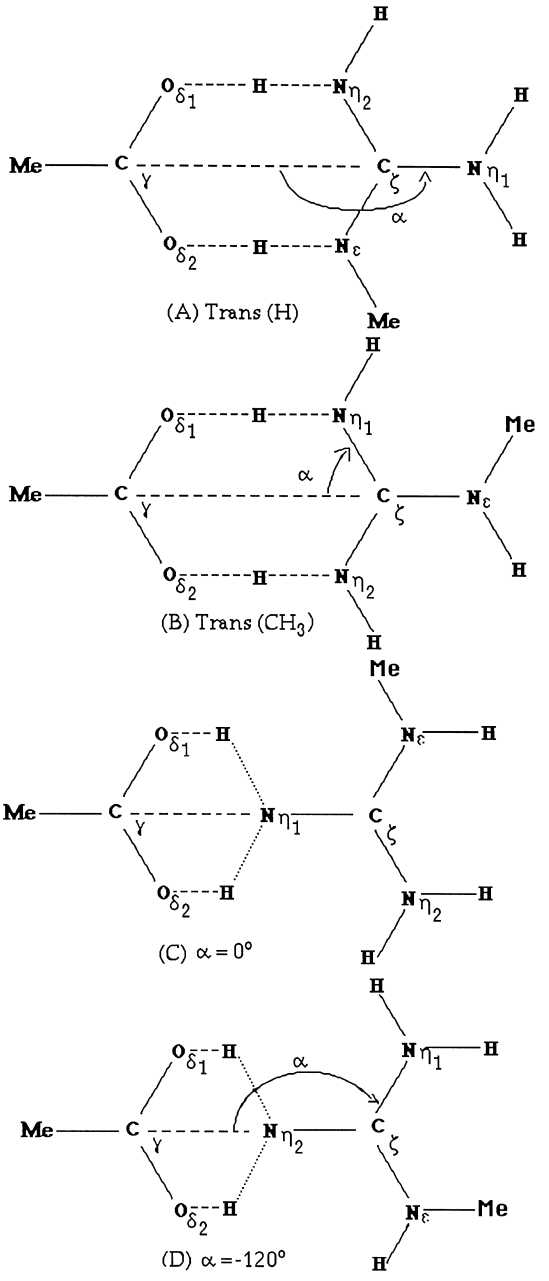

Fig. 2. Schematic representation of conformations where two protons have the same probability to be transferred from the methylguanidinium group to the acetate group.

and between the neutral pair (MG,HAc). This effect should strongly favour the zwitterionic form due to the larger coulombic forces between two opposite charged monomers relatively to that occurring between the polar neutral ones.

In a protein, a third effect, the interaction with the environment, should be considered also for a correct description of the system. Hydrophilic environments should strongly stabilize the charge separation associated to the zwitterionic form. Using their LSCF method, Thery et al. [20] have presented some evidence that confirms this hypothesis. These authors have studied the ASP[69]–ARG[71] interaction in dihydrofolate reductase enzyme. Their calculations indicate that the zwitterionic form is more stable than the neutral form and that the main cause of this preferential stabilization is the interaction with the hydrophilic (protein and water) environment. On the other hand, solvent-free hydrophobic environments should slightly favor the neutral form. However, because the dielectric constant has here values close to 1, it seems reasonable to use in vacuo conditions to simulate these environments.

3. Computational details

In order to study the ARG–GLU and ARG–ASP interactions quantum mechanically as well as the proton transfer mechanism associated with them, the following methodology has been used in our conformational studies of the arginine–carboxylate systems:

- In each point of the conformational space, the rotation angle α was fixed, while the other inter and intra-molecular parameters were optimized (see Fig. 1).
- The study was limited to the conformations in which the heavy atoms of both monomers are in the same plane, because this is the region where most arginine–carboxylate interactions seem to occur [8,14].
- The starting points of the conformational analysis were the trans(H) ($\alpha = 180°$) and the trans(CH_3) ($\alpha = -60°$) conformations. These conformations have been considered to be the most favoured [8,13–18], because they are the only ones where it is possible to establish two interactions of the type (N--H--O⁻⁻C) (see Fig. 2).
- The model for the neutral species was built transferring the most favourable proton from the methylguanidinium group to the acetate group.

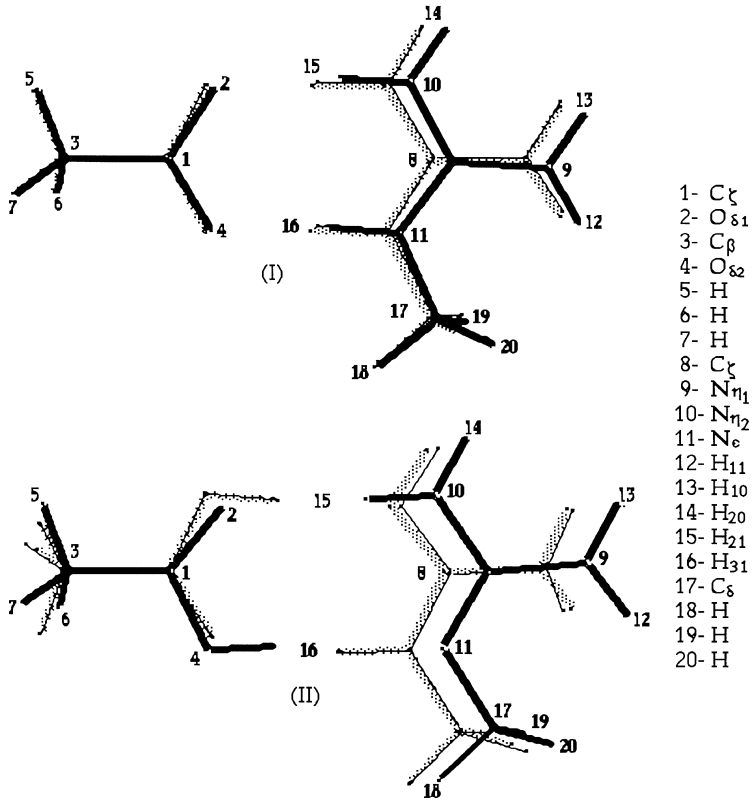

Fig. 3. Optimized geometries of the zwitterionic (I) and neutral (II) forms in trans(H) region, obtained with MP2/6-31G** (▤), 6-31G** (——) and AM1 (——).

We had to decide on this transferral preference. In principle, the pK_a of the arginine guanidinium group should help out deciding on which of the protons should be transferred. The experimental pK_a values have been determined by Schmidt et al. [35] and reported to be equal to 12.5, at 25°C, for all protons of the arginine guanidinium group, i.e. for ϵ-NH as well as η-NH. Even recognizing the high symmetry of the system in question, it does seem unlikely that the pK_a should be exactly identical and there is some mention to the fact in the literature [36]. However, and due to the unconclusive results, we performed some calculations related to this matter — in the conformations (trans(H) ($\alpha = 180°$), trans(CH_3) ($\alpha = -60°$), $\alpha = 0°$ and $\alpha = -120°$) where two protons have the same probability to be transferred (see Fig. 2), the two geometries have been considered. For each case, we selected the geometry with the lower energy; this geometry became our model for the neutral species.

- Each conformation was obtained from the previous optimized one and using increments of 10° in α. In the AM1 studies 36 conformations have been studied for each form, i. e. 72 conformations in total. In the MP2/6-31G** calculations, we have studied 6 conformations for each form, symmetrically distributed relatively to each initial position. We have also performed, for both zwitterionic and neutral forms, less restricted optimizations on the minimum energy conformations of the trans(H) and trans(CH_3) regions. The AM1, 6-31G** and MP2/6-31G** methods were employed and the angle of rotation α was allowed to relax during the optimization procedure to find the global minimum of each form.

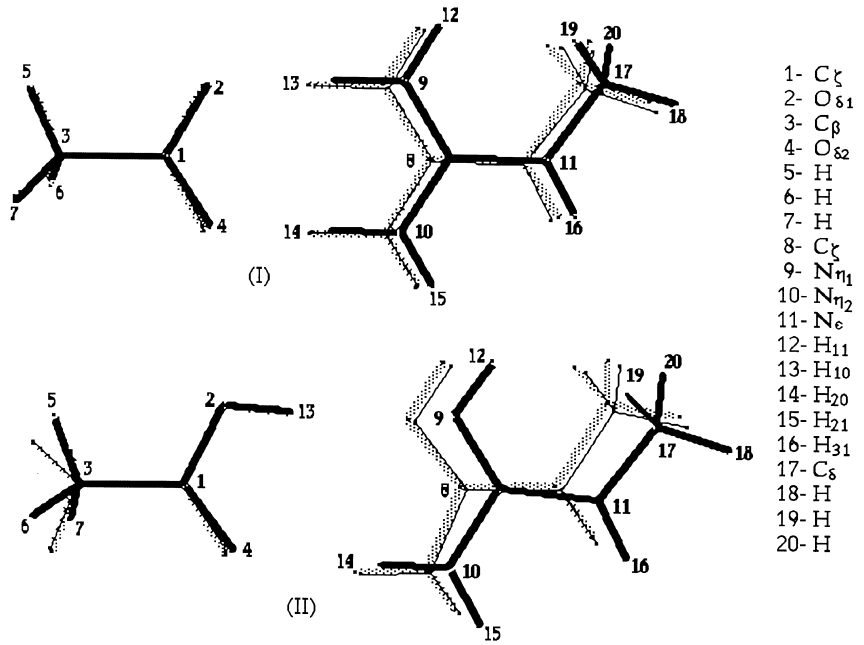

Fig. 4. Optimized geometries of the zwitterionic (I) and neutral (II) forms in trans(CH_3) region, obtained with MP2/6-31G** (▤), 6-31G** (——) and AM1 (——).

- The counterpoise correction [37] was calculated for the global minima ab-initio (6-31G** and MP2/6-31G**) geometries. The basis set superposition error (BSSE) was evaluated to be always less than 1.3% of the correspondent stabilization energy value. Therefore, this correction has been considered of small importance in the present study and was not performed in the remaining calculations.
- The AM1 and ab initio conformational studies have been carried out within the packages AMPAC [38] and GAUSSIAN92 [39], respectively. All the calculations have been performed using an IBM Risc 6000 workstation.

4. Results and discussion

The trans(H) and trans(CH_3) optimized geometries of the zwitterionic and neutral forms are shown in Figs 3 and 4. The most relevant corresponding geometrical parameters are presented in Tables 1 and 2.

Analysis of these results enables us to conclude that the electronic correlation does not introduce any significant enhancement in the values calculated at the 6-31G** level. However, the AM1 results exhibit some differences when compared with the ab initio values. In fact, while the ab initio calculations always transfer the proton to the $O_{\delta 1}$ atom, the AM1 method prefers a proton transfer to the $O_{\delta 2}$ atom in the

Table 1
Most important optimized parameters in the trans(H) region (distances are in angstroms, angles in degrees)

Method	Form	α	$d(C_\zeta–C_\gamma)$	$d(H_{21}–N_{\eta 2})$	$d(H_{21}–O_{\delta 1})$	$d(H_{31}–N_\epsilon)$	$d(H_{31}–O_{\delta 2})$	$d(N_{\eta 2}–O_{\delta 1})$	$d(N_\epsilon–O_{\delta 2})$
MP2/6-31G**	Zwitterionic	180.00	3.83	1.08	1.51	1.09	1.50	2.60	2.59
MP2/6-31G**	Neutral	− 177.73	4.00	1.65	1.02	1.02	1.84	2.68	2.85
6-31G**	Zwitterionic	180.00	3.88	1.04	1.62	1.04	1.62	2.66	2.66
6-31G**	Neutral	− 177.19	4.10	1.81	0.98	1.00	1.96	2.80	2.95
AM1	Zwitterionic	177.10	4.12	1.02	1.83	1.05	1.69	2.85	2.74
AM1	Neutral	− 173.69	4.61	0.99	2.10	2.43	0.98	3.09	3.40

Table 2
Most important optimized parameters in the trans(CH_3) region (distances are in angstroms, angles in degrees)

Method	Form	α	$d(C_\zeta–C_\gamma)$	$d(H_{10}–N_{\eta1})$	$d(H_{10}–O_{\delta1})$	$d(H_{20}–N_{\eta2})$	$d(H_{20}–O_{\delta2})$	$d(N_{\eta1}–O_{\delta1})$	$d(N_{\eta2}–O_{\delta2})$
MP2/6-31G**	Zwitterionic	− 58.19	3.82	1.10	1.47	1.08	1.52	2.54	2.62
MP2/6-31G**	Neutral	− 52.87	4.02	1.68	1.02	1.02	1.88	2.70	2.89
6-31G**	Zwitterionic	− 59.94	3.87	1.05	1.60	1.04	1.61	2.64	2.66
6-31G**	Neutral	− 53.25	4.11	1.84	0.98	1.00	1.99	2.81	2.97
AM1	Zwitterionic	− 59.97	4.12	1.03	1.77	1.03	1.76	2.80	2.79
AM1	Neutral	− 60.04	4.57	2.37	0.98	0.99	2.10	3.34	3.09

trans(H) conformation. The AM1 $C_\gamma–C_\zeta$ distances are always overestimated when compared to the ab initio predictions. The optimized α values obtained at AM1 level are quite different from the corresponding ab initio ones, except for the zwitterionic form in the trans(CH_3) region.

The ab initio N···O distances for the zwitterionic form are shorter than the mean values found in a systematic analysis of the arginine–carboxylate interaction in crystallographic data [8, 12]. In fact, Singh et al. [8] analysed 37 high resolution protein structures and obtained a normal distribution of N···O distances centred around 2.8 Å. In his statistical studies, Gorbitz [12] obtained sample means of 2.888 Å and 2.885 Å for the trans(H) and trans(CH_3) conformations, respectively. Most of the arginine–carboxylate interactions, found in crystallographic protein structures, may occur in hydrophilic environments which means that the zwitterionic form should be more stable than the neutral one. The shorter values obtained for the zwitterionic N···O distances are certainly caused by a stronger interaction between the opposite charged ions in vacuo. The zwitterionic N···O distances predicted by the AM1 method are in good agreement with the experimental values. However, this agreement should result from a poor description of the electrostatic interactions in vacuo given by this method.

The calculated N···O distances for the neutral form should not be directly compared with mean values found in statistical analysis of crystallographic data. These calculated values should be only compared with mean experimental values for the arginine–carboxylate interactions occurring in hydrophobic environments.

The total energies of the monomers are presented in Table 3. Regarding the nature of the arginine–carboxylate interaction, we have mentioned the fact that the relative stabilization energy ($\Delta\Delta E$) between the zwitterionic and neutral forms can be obtained as the difference in their respective energies. This quantity is the result of two opposite effects, one which should favour strongly the zwitterionic form due to coulombic forces and another one which is connected with the relative stability of the charged monomers and the neutral ones. This latter effect cannot be predicted as easily as the former but, in fact, the results shown in Table 3 confirm that the sum of the neutral monomers' total energies (MG,HAc) is lower than the one for the charged monomers (MGH^+,Ac^-). If, on one hand, this difference is relatively low ($\sim$ 500 kJ mol^{-1}) when compared to the energies of the individual monomers, the fact that it takes this same value irrespective of the method used, including AM1, seems to indicate that effectively the set (MG,HAc) is more stable than (MGH^+,Ac^-), favouring the neutral form.

Fig. 5 shows a plot of the energy of stabilization ΔE with the angle of rotation α, obtained using the three theoretical methods. These values are also presented in Table 4. The analysis of these results shows that the

Table 3
Energies of the monomers (kJ mol^{-1}). MGH^+: methylguanidinium. Ac^-: acetate. MG[trans(H)]: methylguanidine originated when a proton is transferred from methylguanidinium to the acetate in the trans(H) conformation. MG[trans(CH_3)]: methylguanidine originated when a proton is transferred from methylguanidinium to the acetate in the trans(CH_3) conformation. HAc: acetic acid

Monomer	MP2/6-31G**	6-31G**	AM1
MGH^+	− 641551.17	− 639521.00	− 94653.93
Ac^-	− 598256.42	− 596592.25	− 90675.06
MG[trans(H)]	− 640488.29	− 638430.72	− 93897.45
MG[trans(CH_3)]	− 640495.81	− 638438.68	− 93883.58
HAc	− 599805.13	− 598147.34	− 91940.80

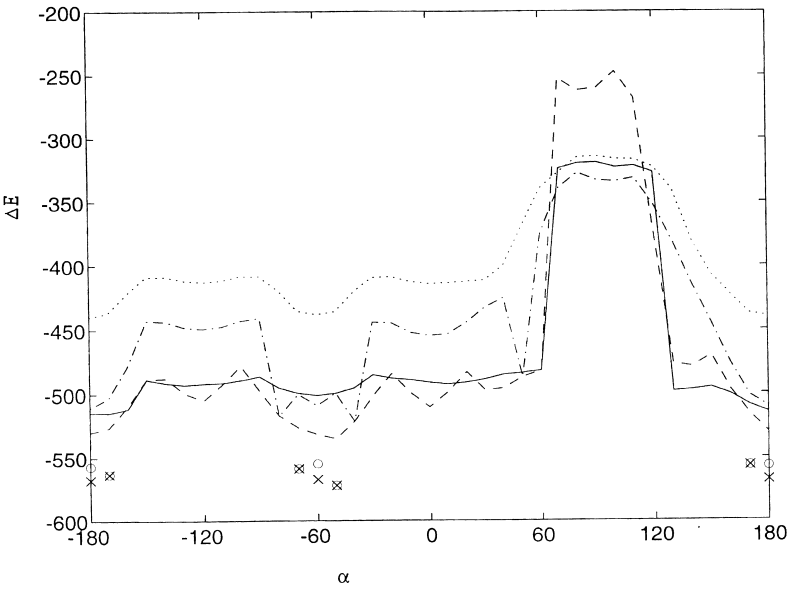

Fig. 5. Plot of energy of stabilization ΔE(kJ/mol) vs. angle of rotation α(degree) for (…) methylguanidinium against acetate, AM1 calculations; (–) methylguanidine against acetic acid, AM1 calculations; (–·) methylguanidinium against acetate, 6-31G** calculations [19]; (- -) methylguanidine against acetic acid, 6-31G** calculations [19]; (o) methylguanidinium against acetate, MP2/6-31G** calculations; (x) methylguanidine against acetic acid, MP2/6-31G** calculations; (⊗) conformations where proton transfer has been observed during the MP2/6-31G** optimization procedure, starting from a zwitterionic initial geometry.

neutral form is more stable than the zwitterion, when a terminal (N–H) proton is transferred from the methylguanidinium to the acetate. In the most unstable conformational region ($\alpha = 70°$ to 120°), an inversion is detected in the general tendency. In that region the methyl group strongly interacts with the acetate and the only protons which can be transferred are those from this group. These protons exhibit poor acidic properties which explains the detected inversion.

The AM1 energy differences between the two mentioned forms are significantly large relative to the ab initio ones. In both minima, the two ab initio (6-31G** and MP2/6-31G**) methods give similar energy differences.

According to all three methods, the minima of the conformational space occur in the region of conformations trans(H) (180°) and trans(CH_3) (−60°), in agreement with all theoretical calculations [14–18] and analysis of structural data [8, 13, 14].

In the AM1 and 6-31G** calculations, two shallow local minima can be observed for the zwitterionic and neutral forms. These points correspond to the conformations (−120° and 0°) where both protons belonging to a same nitrogen are in a position to interact with the acetate group (see Fig. 2).

In the 6-31G** calculations, proton transfer has been detected occurring from the methylguanidinium to the acetate during the optimization procedure. This phenomenon has been observed in three different conformations (−80°, −40° and 50°). In the MP2/6-31G** calculations, proton transfer has been also detected in all conformations around the trans(H) and trans(CH_3) points, during the optimization procedure.

5. Conclusions

Arginine–carboxylate interactions, in proteins, have been associated generally with a zwitterionic state rather than a neutral one [8–18]. The cause of this preferential stabilization has been associated with the strong electrostatic interaction between the two opposite charged ions.

In our studies of this system, both ab initio and semi-empirical (AM1) conformational analysis,

Table 4
Energy of stabilization (kJ mol^{-1}) vs angle of rotation (degree). Zwitter.: zwitterionic form; Neut.: neutral form. [a]α optimized in trans(CH_3) region, the geometrical parameters are presented in Table 2. α optimized in trans(H) region, the geometrical parameters are presented in Table 1. [c]Conformations where proton transfer has been observed to occur from a zwitterionic initial geometry. [d]Conformations where proton transfer has been observed to occur from a neutral initial geometry.

	MP2/6-31G**		6-31G**		AM1	
α	Zwitter.	Neut.	Zwitter.	Neut.	Zwitter.	Neut.
-170	-563.25[c]	-563.25	-502.95	-526.50	-436.62	-496.26
-160			-478.63	-509.56	-421.85	-509.56
-150			-442.92	-488.63	-408.96	-481.22
-140	—	—	-443.67	-488.05	-408.78	-483.06
-130	—	—	-448.02	-499.45	-412.01	-488.50
-120	—	—	-449.11	-504.97	-413.03	-492.07
-110	—	—	-447.53	-492.98	-411.84	-488.08
-100	—	—	-442.92	-478.34	-408.53	-482.20
-90	—	—	-440.85	-497.78	-408.49	-483.35
-80	—	—	-516.83[c]	-516.83[c]	-419.65	-488.66
-70	-558.63[c]	-558.63	-500.25	-526.45	-435.44	-498.83
-60	-554.84	-567.35	-508.92	-531.68	-438.14	-501.30
opt[a]	-554.97	-573.15	-508.92	-536.35	-438.14	-501.30
-50	-572.22[c]	-572.22	-499.65	-535.27	-435.86	-499.73
-40	—	—	-521.40[c]	-521.40	-420.88	-489.86
-30	—	—	-444.33	-501.74	-409.53	-484.10
-20	—	—	-444.89	-484.62	-409.00	-480.65
-10	—	—	-452.33	-499.60	-412.76	-486.76
0	—	—	-454.72	-510.37	-414.47	-491.39
10	—	—	-453.54	-498.86	-413.87	-483.97
20	—	—	-444.51	-483.39	-412.72	-479.45
30	—	—	-432.81	-497.79	-411.66	-481.01
40	—	—	-425.68	-495.93	-400.16	-485.29
50	—	—	-487.38[c]	-487.38	-369.90	-484.09
60	—	—	-375.36	-482.23	-339.74	-482.17
70	—	—	-339.26	-252.76	-327.03	-324.10
80	—	—	-327.64	-262.20	-315.34	-319.94
90	—	—	-333.31	-260.73	-314.50	-315.34
100	—	—	-334.29	-247.47	-316.57	-313.20
110	—	—	-331.48	-267.95	-317.00	-321.94
120	—	—	-351.87	-351.87[d]	-322.66	-326.94
130	—	—	-381.49	-476.88	-340.35	-498.59
140	—	—	-415.34	-479.34	-380.70	-497.38
150	—	—	-444.53	-470.34	-406.88	-495.62
160	—	—	-476.35	-497.31	-422.92	-500.87
170	-556.57[c]	-556.57	-501.74	-515.69	-437.54	-509.28
180	-556.99	-567.81	-510.40	-530.06	-440.02	-514.48
opt[b]	-556.99	-568.25	-510.40	-530.71	-440.22	-514.87

using appropriate molecular models, have been carried out for the above mentioned forms in vacuo.

The ab initio calculations have been performed at accurate 6-31G** and MP2/6-31G** levels. The results obtained with both methods are in good agreement and indicate that, in vacuo, the neutral form should be more stable than the zwitterion. This preferential stabilization should be associated with a larger intrinsic proton affinity of an oxygen atom of the carboxylate group relatively to a nitrogen atom of the guanidinium group. We believe that these results can be extrapolated to hydrophobic environments.

In hydrophilic environments, the zwitterionic form should be more stable than the neutral one. However, in that case, the interaction with the environment is probably more important than the electrostatic interaction between the opposite charged monomers, leading to a preferential stabilization.

Our calculations also indicate that the preferred conformations are those where it is possible to establish two interactions of the type (N--H--O=C), which is in agreement with previous calculations [14–18] and statistical analysis of the crystallographic data [8, 13, 14] available.

Despite the differences found between the semi-empirical and ab initio results, AM1 calculations could be a reasonable first approach to study arginine–carboxylate interactions in very big systems. In fact, the inclusion of the protein environment and of explicit water molecules around the interacting pair, to take into account the influence of the first hydration shell molecules, can be very important in achieving a correct description of this interaction in hydrophilic medium. However, this can be a very time consuming task at ab initio level, and, therefore, the semi-empirical calculations may constitute a reasonable alternative strategy.

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