Ab-Initio Calculations on Arginine−Disulfide Complexes Modeling the One-Electron Reduction of Lysozyme. Comparison to an Experimental Reinvestigation

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Received: October 24, 1996; In Final Form: March 12, 1997[⊗]

The one-electron reduction of hen egg white lysozyme has been reinvestigated by γ-radiolysis using $CO_2^{\bullet-}$ free radicals as reductants. We show that the reaction is specific toward one out of the four disulfide bridges, i.e. the 6−127 one. This bond is in interaction with the charged end of arginine 5. The reduction leads to thiol functions and to a lesser extent to fragmentation of the polypeptide chain, which can only come from electron migration from disulfide. To get a better insight into the mechanism induced by electron transfer to the protein, the 6−127 disulfide bridge and the charged end of arginine 5 in lysozyme were modelized by R_2S_2 (R = H, CH_3) and $C(NH_2)_3^+$, and *ab-initio* calculations were performed. All separate molecular and radical entities resulting from the electron addition were optimized with two basis sets (6-31G* and 6-31+G*) and at the MP2 correlation level. The formation of complexes was studied and four zwitterionic and two neutral radical complexes involved in charge transfer reaction were characterized at the MP2 level. The influence of the environment was taken into account by using the Onsager reaction field method (SCRF) for the isolated species as well as the complexes.

Introduction

A number of recent studies have demonstrated that reduction/oxidation reactions of sulfur residues in proteins are intimately involved in the control of vital processes such as signal transduction, gene expression, and regulation of proliferative events.[1] These groups function by thiol−disulfide exchange reactions which are one- or two-electron oxido-reductive processes. The free radical chemistry of sulfur compounds thus continues to receive much attention.[2−5] It is known that disulfide groups are able to trap excess free electrons initially localized at other groups in proteins, acting as electron sinks.[6] We are currently studying by γ-radiolysis and pulse radiolysis the mechanism of the reductive cleavage of disulfide bonds in several proteins[7−9] in order to explore the role of the three-dimensional structure of the protein and the sulfur environment, in its redox properties. The reducing agent is carboxyl radical, which reacts selectively with disulfide bonds in proteins. We show indeed that the reactivity of these SS bonds is strongly dependent on their amino acid environment and solvent accessibility. Therefore as a model we have reinvestigated the one-electron reduction of lysozyme[9] since all previous studies were incomplete.[10−13] Lysozyme has four disulfide bonds, positioned respectively at 6−127, 30−115, 64−80, and 76−94 residues, and no thiol functions. The three-dimensional structure is known in the crystal phase[14] as well as in aqueous solution.[15−16] We present here the characterization of the only reactive disulfide bond by the free radical reductants $CO_2^{\bullet-}$, and we show that this reaction can lead to two types of final compounds, i.e. thiol groups resulting from direct disulfide reduction and fragmentation, which can only result from electron migration from a sulfur function. Our aim was then to provide possible explanations for the reactivity observed. For this purpose we used the methods of quantum chemistry to investigate the importance of the neighboring amino acid on the stability of the one-electron-reduced disulfide and the influence of the environment. Part of these results have been presented in a preliminary form.[9,17]

Experimental Section

1. Materials and Methods. *Reagents.* Formic acid, sodium formate, KH_2PO_4, and K_2HPO_4 were of the highest purity available (Merck, Prolabo) and used as received. *N*-Iodoacetamide and β-maleimidopropionic acid were from Sigma. Water was purified using a Spectrum system and subsequently distilled in a quartz apparatus.

Lysozyme. Hen egg white lysozyme (Sigma) was purified to homogeneity by ion exchange chromatography (20 mM Tris-HCl buffer, pH 7.8, 400−1000 mM NaCl linear gradient) over Macro-Prep High S resin (Bio-Rad) followed by concentration, gel filtration over Sephadex G-75 (10 mM phosphate buffer, pH 7.0), total dialysis against distilled water, and lyophilization. Purity was checked by homogeneous 12.5% polyacrylamide gel electrophoresis with 0.2% lauryl sulfate. The concentration of protein was measured spectrophotometrically[18] using $\epsilon_{278} = 37.9$ mM^{-1} cm^{-1}. Radiolytically reduced lysozyme for nuclear magnetic resonance analysis was prepared as follows: 50 mL of 250 μM lysozyme in 20 mM phosphate, 100 mM formate buffer, pH 7.0, was saturated with N_2O and irradiated to 125 Gy with γ -rays (4.2 Gy min^{-1}) in a 100 mL Erlenmeyer flask stoppered with a rubber septum. The free thiol:protein molar ratio at this stage was titrated at 2:1. A 2-fold molar excess, relative to titrable thiols, of β-maleimidopropionic acid in 20 mM phosphate buffer, pH 7.0, was then introduced using a syringe. Complete alkylation of thiols was reached in less than 30 ms. The solution was then dialyzed against 20 mM Tris-HCl buffer, pH 7.8, centrifuged and purified by ion exchange chromatography in the same way as for the native protein. S-carboxylated lysozyme, coming off at 550 mM NaCl, was easily separated from native protein peaking at 870 mM NaCl, and from minor amounts of reaction by-products.

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[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1997.

S1089-5639(96)03312-9 CCC: $14.00

Titrations. Free sulfhydryl groups were determined by optical titration with 5,5′-dithionitrobenzoic acid at pH 8.0 (100 mM Tris-HCl buffer), using $\epsilon_{410} = 13.6$ mM^{-1} cm^{-1} for the 3-carboxylato-4-nitrothiophenolate anion.[19] Thiol groups in reduced protein were routinely alkylated prior to electrophoresis by reaction with 10 mM iodoacetamide (10 min, room temperature).

Lysozyme Enzymatic Activity. The catalytic activity of lysozyme was measured with *Micrococcus lysodeikticus*, as described by Shugar.[20]

γ-Radiolysis Experiment. γ-Irradiations were performed with a ^{60}Co source at a dose rate between 4.25 and 21 Gy min^{-1}. The dosimetry was performed by the Fricke's procedure. Solutions to be irradiated were made up in 20 mM dipotassium hydrogen phosphate and contained sodium formate. pH was adjusted with formic acid. The samples were equilibrated with N_2O by flushing for at least 60 min in dim light under permanent stirring or vortexing, thus avoiding bubbling in the solution. The reducing free radicals are created upon scavenging of the water free radicals by the system [formate + N_2O].[3] In γ-radiolysis, a steady state is created that lasts as long as the irradiation does. The radiolytic yield is 0.62 μmol J^{-1}.

NMR Analysis. Lysozyme samples were prepared in 90% H_2O, 10% D_2O without buffer at a concentration of 3 mM. The data were acquired at 35 °C and pH 3.8 as complete ^{1}H assignments for native lysozyme in these conditions were available.[15] The identification of the amino acid residues affected by γ-irradiation was provided by the measurements of changes in chemical shifts. ^{1}H NMR spectroscopy was performed at 500 MHz on a Varian Unity 500. Standard methods were used to obtain pure absorption DQF-COSY.[21] The data processing was performed using the VNMR software package. NMR spectra were recorded using the Varian Unity 500 device.

Electrophoresis. Homogeneous 12.5% polyacrylamide gel electrophoreses in the presence of sodium dodecyl sulfate (2%) (SDS-PAGE) were performed to analyze the products of radiolysis. Thiol groups were blocked by 10 mM iodoacetamide (Serva) immediately after radiolysis. For disulfide bond characterization, reduction was realized by β-mercaptoethanol.

2. Results and Discussion. Aqueous solutions of lysozyme (250 μM to 2 mM, phosphate buffet 0.02 M) were irradiated in the presence of formate (0.1 M) and in an atmosphere of N_2O (dose rate = 4.29 or 21 Gy min^{-1}). pH varied between 5 and 10.

Figure 1 shows the variation of the amount of thiol groups as a function of the dose for irradiations at pH 5 and for various lysozyme initial concentrations. Similar experiments were performed at pH 6−10. At all pHs, the stoichiometry of the reaction is two thiol groups per one lysozyme, indicating that only one disulfide group is broken. The initial yield of the reduction is strongly pH dependent and always higher than that of $CO_2^{\bullet-}$ since it has been shown that it is a chain reaction in small disulfides[22,23] as well as in proteins.[7]

Analysis by SDS-PAGE (Figure 2) reveals that polymers and fragments are created by irradiation. For instance at 25.8 Gy, the major bands are native lysozyme and dimer, but a polymer not corresponding to a multiple of lysozymes is observed (ca. 24 000 Da) and also a band at ca. 11 500 Da. After reduction by β-mercaptoethanol, the dimers disappear and the band corresponding to lysozyme is increased. This shows that these dimers were linked by intermolecular disulfide bonds. Confirmation of the cleavage of one of the four disulfide bridges of lysozyme and identification of its sequence position have been provided by ^{1}H nuclear magnetic resonance spectroscopy.

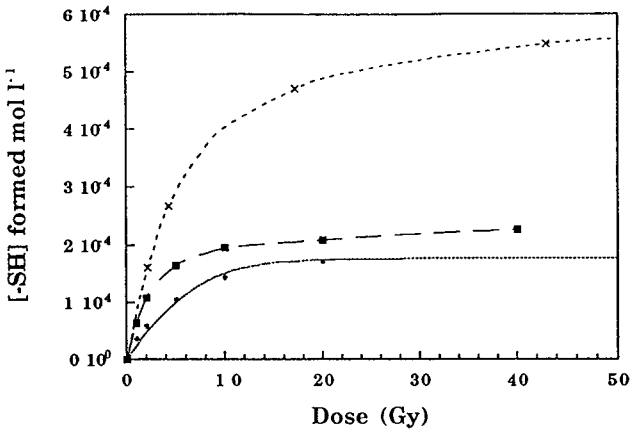

Figure 1. Reduction of lysozyme by $CO_2^{\bullet-}$ free radicals produced by γ-radiolysis. Variation of the amount of thiol functions created as a function of the dose. Formate 0.1 M, phosphate 10 mM, pH 5, I = 4.25 Gy mn^{-1}: (×) [lysozyme] = 2.6 × 10^{-4} M; (■) [lysozyme] = 1 × 10^{-4} M; (◆) [lysozyme] = 0.8 × 10^{-4} M.

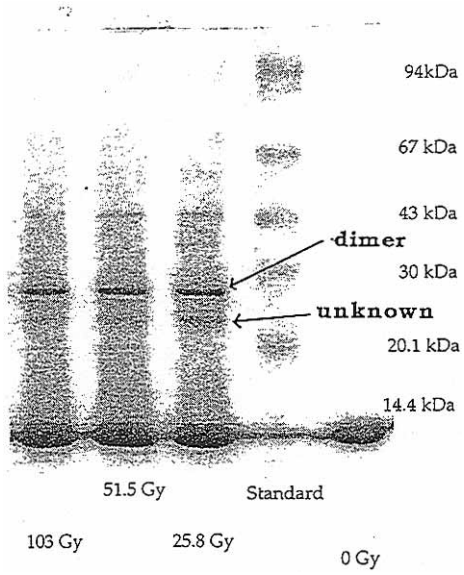

Figure 2. SDS-PAGE analysis of lysozyme nonirradiated (0 Gy) and reduced by $CO_2^{\bullet-}$ free radicals with various doses. [lysozyme] = 250 M, formate 0.1 M, phosphate 20 mM, N_2O, pH 5, I = 4.29 Gy min^{-1}. The arrows indicate respectively the dimer (28 kDa) and a mixed disulfide made of lysozyme and a fragment (ca. 24 kDa) noted "unknown".

One- and two-dimensional spectra of the modified lysozyme have been compared with data for the wild type protein under similar conditions. The 1D spectrum of each protein is shown in Figure 3.

The close similarity between the two spectra indicates that the overall three-dimensional structure of lysozyme is well preserved after γ-irradiation. Analysis of the backbone C_αH-NH region of the two-dimensional COSY spectrum of each protein (Figure 4) supports this observation. Some differences in the two spectra are however noticeable. Indeed, the Cys6 and the Cys127 resonances and those of the immediate sequential neighbor of these two amino acids display significant deviations in chemical shifts, as shown in Figure 4.

These modifications undoubtedly result from conformational or chemical changes in the corresponding polypeptide region following the radiolytic reduction of the disulfide bond. On the contrary, the resonances of the remaining cysteines have still identical chemical shifts, confirming that only the disulfide bridge linking Cys6 and Cys127 has been broken. This cleavage seems to produce no dramatical effect on the overall three-

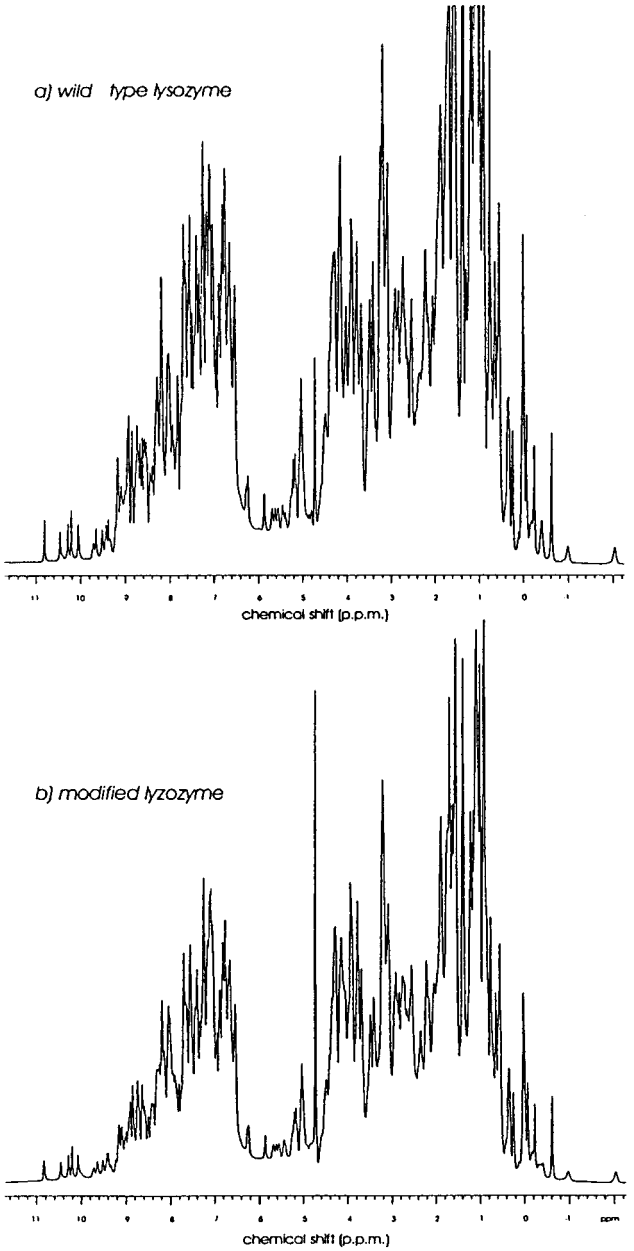

Figure 3. 500 MHz ^{1}H NMR spectra of (a) wild type and (b) modified lysozyme.

dimensional structure of lysozyme as it was described.[24] Indeed we have verified that the enzyme is not inactivated by γ-radiolysis reduction. It has been shown that the 6−127 bond is specifically reduced for ubiquitin conjugation[25] and by dithiothreitol.[24] Examination of the structure of the protein shows that this bond is the most exposed to the solvent and hence the most accessible to reactants. In addition it is in interaction with the positively charged end of arginine, which may facilitate the guidance of carboxyl radical to this site. The other disulfide bridges are surrounded by aromatic amino acids. We thus propose that the reaction does take place on the 6−127 bond. However it is known that long-range intramolecular electron transfer occurs in proteins;[26,27] thus the final localization of the electron might not be the attacked residue. To understand why the electron can be localized at the 6−127 bridge, we have undertaken investigation of the thermodynamic stability of one-electron-reduced disulfide in interaction with the charged end of arginine. So we have modelized the 6−127 disulfide bridge by RSSR, R = H and CH_3, and arginine 5 by the guanidinium ion, $C(NH_2)_3^+$, and we have performed some theoretical *ab-initio* calculations on these compounds, their radical derivatives, and the complexes they can form. Although these model compounds present little interest in terms of their biological potential, their properties can aid in understanding the reactivities of other disulfide-containing compounds.

Theoretical Section

We have first considered all possible species coexisting before and after the one-electron addition. From a theoretical point of view it has appeared interesting to calculate the electronic affinities (EAs) of separated entities $C(NH_2)_3^+$, $RS^\bullet$, and R_2S_2 and the binding dissociation energy (BDE) of the SS bond in $R_2S_2^{\bullet-}$. The obtained values are compared when possible to recent experimental and theoretical results.[28−33] In a second step we have studied cationic complexes, $C(NH_2)_3^+\cdots R_2S_2$, and radical ones resulting from addition of one electron. Effects of the environment on the EA values and complexation energies of the equilibrium structures already optimized in vacuum at the MP2 level are investigated by employing the self-consistent reaction field (SCRF) model.

1. Calculation Methods. In recent years several *ab-initio* calculations were performed on RSSR molecules.[34−36] Use of an extended basis set was of a prime importance to treat correctly the sulfur compounds. More recently several studies were undertaken on ions and radical sulfur derivatives with sophisticated bases, including correlation effects.[32,37−45] *Ab-initio* calculations have been performed, respectively, at restricted and unrestricted Hartree−Fock (RHF, UHF) levels for open and closed shell systems and at second-order Moller−Plesset (MP2) levels using two basis sets, 6-31G* and 6−31+G*. The addition of diffuse functions is generally required for proper description of anionic species.[46] UHF and UMP2 gradient geometry optimizations of the entities under consideration were done using both basis sets. The correlation effects were systematically included in our study, because it is well-known that in the reactions involving possible neutral and zwitterionic complexes the energy difference between these two complexes is strongly sensitive to these effects.[47,48] Single-point calculations at the MP4(STDQ) level of theory with the 6-31G* basis set have been performed on UMP2/6-31G* optimized radical complexes to refine the energy differences. Since the doublet wave function (UMP2, UMP4) is not necessarily an eigenfunction of the S^2 operator, some spin contamination appears in the radicals. The small spin contamination of the radicals treated here has been corrected by use of a projection operator on the lowest state (PUMP2 and PUMP4). Zero-point vibrational energy (ZPE) was taken into account for some essential points at the MP2/6-31G* and MP2/6−31+G* levels. Variations of net atomic charges were investigated with the help of Mulliken population analysis, which remains quite useful even if it does not perfectly represent the charge density distribution. To account for possible effects of the environment on the model compounds, we used the Onsager reaction field method (SCRF). In this model, the ionic and neutral compounds are immersed in a continuous medium of desired dielectric constant. Furthermore, to account for the ion−dipole interaction, a Born charge term is added to the energy. To mimic the effect of environment, two different dielectric constants were chosen, $\epsilon = 78$ for the aqueous solvent and $\epsilon = 2$ for the protein environment, as it was proposed for DNA stacked pairs.[43b,c] It is important to note that dispersion was not accounted for in these calculations because of computational limitations. Furthermore, this model places the solute in a spherical cavity and assumes a localized charge at its center, while a cavity that follows the molecular shape with a delocalized charge distribu-

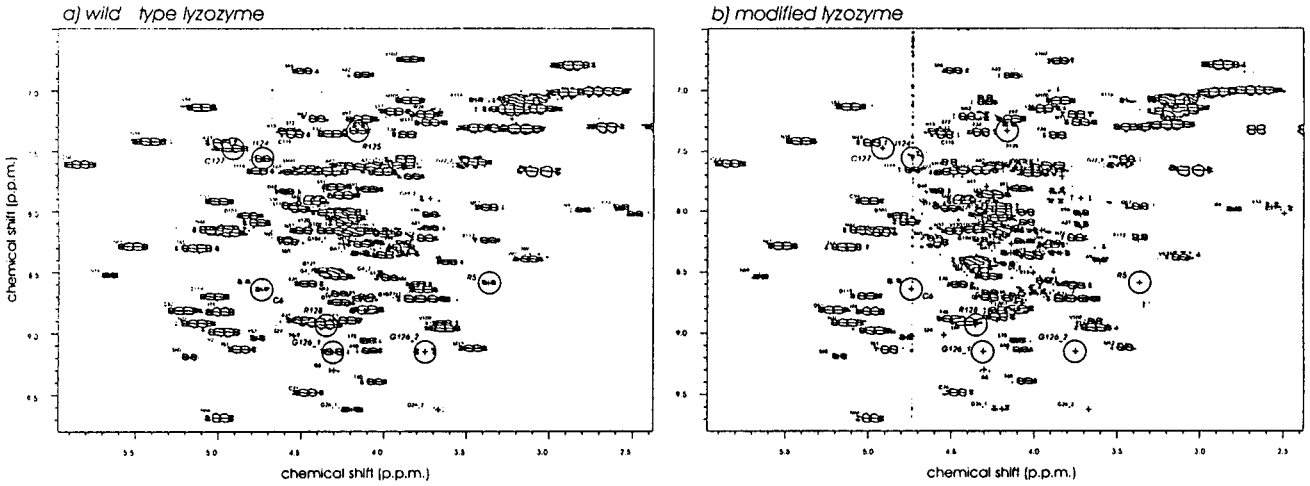

Figure 4. Fingerprint of the two-dimensional COSY spectra of (a) wild type and (b) modified lysozyme. Most of the peaks here result from the interaction between the hydrogen atoms attached to the nitrogen and C_α atoms within one amino acid of the protein. The number is the residue sequence position. Chemical shifts of the peaks in circles are the most affected after γ -irradiation.

tion would be more realistic. Therefore, these results constitute crude initial estimates of the effects of the environment. All *ab-initio* calculations were performed with the Gaussian94 molecular orbital packages[49] on the CRAY C98 at the IDRIS computing center in Orsay (France).

2. Results and Discussion. *a. Isolated Species.* We have tested the validity and limits of the calculation methods for the one-electron reduction of isolated species. So we were interested not only in RSSR and guanidinium radicals but also in RS$^\bullet$ compounds, which were recently studied both experimentally and theoretically.[32,33−36,43] To complete this study, we included the RSS$^\bullet$ radical and RSS$^-$. All the radical sulfur entities were observed.[28−30] As pointed out by Colson and Sevilla,[43a] calculations of electronic affinities (EAs) can be critical, so a full geometry optimization was carried out to obtain adiabatic EA.

i. Sulfur Compounds. Table 1 gives the optimized geometrical parameters and energies of HS$^\bullet$, HS$^-$, HS$_2^\bullet$, HS$_2^-$, H_2S_2, and $H_2S_2^{\bullet-}$ resulting from calculations using two basis sets and MP2 correlation. The corresponding values for the methylated species are reported in Table 2.

An analysis of the influence of the level of calculations and of the substitution on the SS bond has pointed out two main features.

(1) The *geometrical parameters* present little sensitivity to inclusion of diffuse orbitals in the basis set. In agreement with the experimental and theoretical studies, the perpendicular conformation is the most stable for RSSR,[34−36] with a SS bond length varying between 2.063 and 2.072 Å when R = H and decreasing to a stable value of 2.055 Å when R = CH_3. In HSS$^\bullet$, this value is reduced to 1.98−2.00 Å. Conversely, upon addition of an electron, we principally obtain a lengthening of the SS bond from 2.788 to 2.877 Å when R = CH_3 (a slightly smaller variation when R = H), whereas the valence angles, dihedral angles, and other bond lengths remained at their values in the RSSR molecule. The SS bond becomes a three-electron bond, and its length may be compared with a one-electron bond, as in $SO_2-SO_2^-$.[37] The SH bond length is nearly the same for all the entities, around 1.33−1.34 Å, with a small lengthening (1.34−1.35 Å) in HS$^-$ because of the excess of electron localization on the sulfur atom.

(2) While the *electronic affinities (EAs) and bond dissociation energies (BDEs)* are very sensitive to taking into account the correlation effects, which lead in all cases to increased values, only EAs depend on the inclusion of diffuse orbitals. Nevertheless these values remain underestimated compared to the known recent experimental and theoretical data: EA = 1.77 eV for HS$^\bullet$ and 1.34 eV for $CH_3S^\bullet$ in MP2/6-31+G* calculations against respectively 2.31 eV (HS$^\bullet$)[32] and 1.861 eV ($CH_3S^\bullet$).[32,44−45] We have also shown the importance of the ZPE corrections (Table 3), which is more evident for EA than for BDE and strongly depends on the species.

All the sulfur entities (molecular, ionic, and radical) were found to be stable, in agreement with experiments,[7−13,29,30] and the slightly destabilizing effect of methyl substitution on EA is well reproduced for RS compounds cited above as well as for R_2S_2 ones. For these last species, EA values are very small, positive for R = H (between 0.10 and 0.45 eV, depending to the basis sets [Table 1]) and negative for R = CH_3 (between −0.45 and −0.08 eV [Table 2]), so direct addition of one electron on the SS bond does not seem so easy. But taking

TABLE 1: Structures and Energies of the H_2S_2 and Radical and Anion Derivatives with the Different Basis Sets: All Distances are in Angstroms, Angles Are in Degrees, and Total Energies Are in Atomic Units; Electronic Affinities (EA) and Bond Dissociation Energies (BDE) Are in Electronvolts

		6-31G*	MP2/6-31G*	6-31+G*	MP2/6-31+G*
HS$^\bullet$	r_{SH}	1.330	1.344	1.330	1.345
	E	−398.06440	−398.16330	−398.06519	−398.16457
	EA	0.78	1.28	1.13	1.77
HS$^-$	r_{SH}	1.342	1.353	1.340	1.352
	E	−398.09322	−398.21045	−398.10689	−398.22960
HS$_2^\bullet$	r_{SS}	2.000	1.982	2.001	1.984
	r_{SH}	1.329	1.329	1.329	1.348
	θ_{HSS}	100.9	102.1	100.7	101.9
	E	−795.58710	−795.80977	−795.588300	−795.81280
	EA	0.856	1.16	1.07	1.48
HS$_2^-$	*rSS*	2.116	2.110	2.115	2.112
	r_{SH}	1.334	1.351	1.332	1.349
	θ_{HSS}	102.0	102.9	101.6	102.2
	E	−795.61855	−795.85237	−795.62769	−795.86702
H_2S_2	r_{SS}	2.063	2.070	2.065	2.072
	r_{SH}	1.327	1.344	1.328	1.344
	θ_{HSS}	99.0	99.0	99.0	98.9
	ϕ	90.0	90.4	90.1	90.0
	E	−796.17439	−796.41134	−796.175603	−796.41457
	EA	0.18	0.10	0.38	0.45
	BDE	1.24	2.31	1.23	2.32
$H_2S_2^{\bullet-}$	r_{SS}	2.871	2.830	2.876	2.810
	r_{SH}	1.331	1.344	1.331	1.345
	θ_{HSS}	87.4	85.8	88.0	87.7
	ϕ	98.4	100.6	97.7	98.2
	E	−796.18088	−796.41509	−796.189677	−796.43097
	BDE	0.63	1.12	0.48	1.00

TABLE 2: Structures and Energies of $(CH_3)_2S_2$ and Radical and Anion Derivatives with the Different Basis Sets: All Distances Are in Angstroms, Angles Are in Degrees, and Total Energies Are in Atomic Units; Electronic Affinities (EA) and Bond Dissociation Energies (BDE) Are in Electronvolts

		6-31G*	MP2/6-31G*	6-31+G*	MP2/6-31+G*
$CH_3S^•$	r_{SC}	1.809	1.801	1.809	1.801
	r_{CH}	1.081	1.090	1.805	1.092
	E	−437.10151	−437.33233	−437.10249	−437.33524
	EA	0.36	0.91	0.67	1.34
CH_3S^-	r_{SC}	1.832	1.827	1.834	1.834
	r_{CH}	1.091	1.100	1.089	1.099
	E	−437.11486	−437.36595	−437.12693	−437.38461
$(CH_3)_2S_2$	r_{SS}	2.053	2.055	2.054	2.056
	r_{SC}	1.816	1.815	1.815	1.815
	r_{CH}	1.083	1.093	1.081	1.091
	θ_{CSS}	103.0	102.1	103.1	102.1
	ϕ	90.0	90.0	87.2	84.0
	E	−874.24764	−874.75312	−874.24973	−874.76052
	EA	−0.40	−0.45	−0.22	−0.08
	BDE	1.21	2.41	1.22	2.45
$(CH_3)_2S_2^{•-}$	r_{SS}	2.877	2.804	2.874	2.788
	r_{SC}	1.820	1.816	1.820	1.819
	r_{CH}	1.088	1.095	1.084	1.095
	θ_{CSS}	92.9	89.2	92.8	88.6
	ϕ	85.1	94.4	95.8	93.5
	E	−874.23304	−874.73729	−874.241575	−874.75739
	BDE	0.45	1.06	0.33	1.10

TABLE 3: Energies and Adiabatic Electronic Affinities without and with the ZPE Corrections for Some Isolated Species within MP2/6-31G* and MP2/6-31+G* Calculations: Total Energies Are in Atomic Units; Electronic Affinities (EA) and Bond Dissociation Energies (BDE) Are in Electronvolts

	MP2/6-31G*	+ZPE correction	MP2/6-31+G*	+ZPE correction
$HS^•$	−398.16330	−398.15698	−398.16457	−398.15826
HS^-	-398.21045	−398.20435	−398.22960	−398.22349
EA	1.28	1.29	1.77	1.77
H_2S_2	−796.41134	−796.39220	−796.41457	−796.39549
BDE	2.31	2.13	2.32	2.15
$H_2S_2^{•-}$	−796.41509	−796.39983	−796.43097	−796.41557
BDE	1.12	1.05	1.00	0.92
EA	0.10	0.21	0.45	0.55
$(CH_3)_2S_2$	−874.75312	−874.67290	−874.76052	−874.67812
$(CH_3)_2S_2^{•-}$	−874.73729	−874.65970	−874.75739	−874.67749
EA	−0.45	−0.36	−0.08	−0.02
$C(NH_2)_3^+$	-205.12004	−205.03189	−205.12974	−205.04205
$C(NH_2)_3^•$	−205.22454	−205.13806	−205.25229	−205.16718

into account the ZPE corrections [Table 3] leads to higher EA values (for instance the corrected EA of HSSH is 0.55 eV and that of $(CH_3)_2S_2$ is −0.02 eV at the MP2/6−31+G* level). So, we cannot exclude the ability of RSSR to trap an electron. Solvation effects were investigated within the MP2/6-31+G* calculations employing the Onsager model with the dielectric constant of 78 on the equilibrium structures optimized in vacuum. These effects added to the Born charge term substantially stabilize the anions, while the neutral systems are only slightly affected because of the small values of the Born charge term. The combined variations lead to important trends in EA (respectively 3.92 and 2.28 eV versus 1.77 and 0.45 eV for HS and H_2S_2, see Table 4).

Morever, in agreement with experiments, the other possible mechanism consisting of a combination of $RS^•$ and RS^- is pointed out, and we confirm the thermodynamical ability of formation of $R_2S_2^{•-}$.[10,32] However for both substituents the radicals are more stable than the corresponding fragments: a crude evaluation of the bond dissociation energy is about 1 eV (≃23 kcal/mol) at the MP2 level for both $RSSR^{•-}$. It can be noted that the addition of an electron weakens the SS bond; indeed the BDE of RSSR is estimated at 2.31 eV (≃53 kcal/mol) from the recombination of two $RS^•$, a value to be compared to the estimated experimental one of 66.8 kcal/mol[45]). Taking into account the ZPE correction, which gives more accurate values for binding energies,[50] the BDE values are reduced to 2.15 eV for H_2S_2 and to 0.92 eV for $H_2S_2^{•-}$ (Table 3).

TABLE 4: Energies of Isolated Species and of ZW2 and N4 Complexes in Different Continous Media Using the SCRF Method for MP2/6-31+G* Calculations: Total Energies Are in Atomic Units, Electronic Affinities (EA) Are in Electronvolts, and Relative Energies (E_r, Defined with Regards to ZW2) are in kcal/mol

	vacuum	ϵ =78	ϵ = 2
H_2S_2	−796.41457	−796.41562	−796.41496
$H_2S_2^{•-}$	−796.43097	−796.49959	−796.46414
Born charge term		−0.070787	−0.035852
EA	0.45	2.28	1.33
$C(NH_2)_3^+$	−205.12974	−205.20545	−205.16809
Born charge term		−0.07571	−0.03835
$C(NH_2)_3^•$	−205.25229	−205.24669	−205.25195
EA	3.40	1.12	2.28
ZW2	−1001.72450	−1001.74099	−1001.72867
N4	−1001.68786	−1001.69246	−1001.68844
E_r	23.0	36.0	25.2

TABLE 5: Structures and Energies of the $C(NH_2)_3^+$ Derivatives with the Different Basis Sets: All Distances Are in Angstroms, Angles Are in Degrees, and Total Energies Are in Atomic Units; Electronic Affinities (EA) Are in Electronvolts

		6-31G*	MP2/6-31G*	6-31+G*	MP2/6-31+G*
$C(NH_2)_3^+$	r_{CN}	1.322	1.335	1.322	1.336
	r_{NH}	0.997	1.012	0.997	1.013
	θ_{NCH}	121.6	121.6	121.6	121.7
	E	−204.52154	−205.12004	−204.52500	−205.12974
	EA	2.84	2.89	3.33	3.40
$C(NH_2)_3^•$	r_{CN}	1.423	1.425	1.419	1.414
	r_{NH}	1.000	1.019	1.000	1.020
	θ_{NCH}	116.0	113.9	116.3	112.8
	E	−204.62507	−205.22454	−204.63861	−205.25229

ii. Amino Compounds. Table 5 gives the optimized geometries and energies for $C(NH_2)_3^+$ and $C(NH_2)_3$. Geometries are sensitive to the one-electron reduction.

As expected, $C(NH_2)_3^+$ is planar (symmetry group D_{3h}). CN and NH bond lengths vary respectively from 1.322 to 1.336 Å and from 0.997 to 1.013 Å.

The main effect of electron addition is the symmetry change of the corresponding radical $C(NH_2)_3^•$, which becomes pyramidal (C_{3v}) with a longer CN bond, from 1.414 to 1.425 Å. The guanidinium cation has a great ability to trap an electron, with an EA varying from 2.82 to 3.33 eV (from 2.89 to 3.40 eV with the ZPE correction).

Solvation effects were estimated using the Onsager−Born model in the same manner as for the sulfur compounds; the stabilization energy of the cation leads to a reduced EA value (1.12 eV versus 3.33 eV; see Table 4). At this stage of the work, several conclusions are drawn.

Both 6-31G* and 6-31+G* give coherent results for geometrical parameters as well as for energies, but, as was expected, introducing correlation effects is the decisive factor in order to get a semiquantitative agreement.

All the species studied (molecules, radicals, and ions) are stable, as shown experimentally for sulfur derivatives. However the effect of methyl substitution on sulfur compounds is generally minor even on the electron affinity of RSSR, the value of which is very small.

As pointed out in Tables 1, 2 and 5, EA values are very different in vacuum for disulfides and guanidinium group, respectively 0.45 and 3.33 eV, and they strongly depend on the

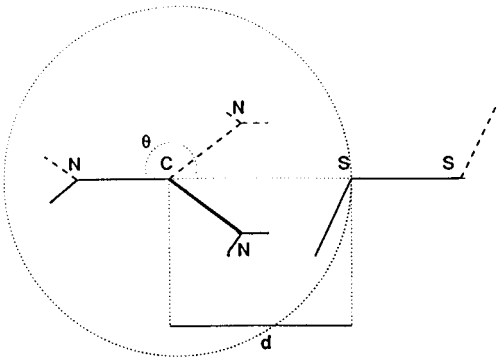

Figure 5. Definition of d and θ describing the relative positions of the guanidinium ion and the disulfide.

medium, which is roughly represented by the dielectric constant in the Onsager−Born method (Table 4): (i) Full solvation of both entities ($\epsilon = 78$) appears to favor disulfides for electron capture, the EA values being now 2.28 and 1.12 eV; (ii) conversely, when these model compounds are surrounded by a nonpolar environment ($\epsilon = 2$), the Born charge term is reduced by about half, and EA values amount respectively to 1.33 and 2.28 eV, favoring the guanidinium group. So, even with these crude estimates, it can be noted that the effects of the medium are of prime importance for the isolated species.

b. Complexes. This analysis of the separated species allows us to investigate now the interactions between disulfide entities and guanidinium ion, before and after electron addition, so we have studied cationic and radical complexes. Once again taking into account both intra- and intercorrelation effects is essential in our study. So as a reasonable approximation, and because calculations at the MP2 level are very time-consuming, we considered only the complexes with R = H. Calculations were carried out with both 6-31G* and 6−31+G* bases sets. Basis set superposition error (BSSE) was corrected by the counterpoise method.[51] Furthermore single-point calculations have been performed within the SCRF method on the most stable UMP2/6-31+G* optimized radical complexes to study the influence of environment.

i. Cationic Complexes. As a first step, a scan was performed, varying two intermolecular geometrical parameters: d, which is the distance between the central atom C of the guanidinium ion and one S of the disulfide molecule, and θ, the angle between the NC bond and the CS direction, defining the relative position of both entities (Figure 5), the other intra- and intermolecular geometrical parameters being fixed to their optimized values (Tables 1 and 4).

Then, geometries of $C(NH_2)_3^+\cdots S_2H_2$ complexes were fully optimized. One main stable configuration was found with a stabilization energy of 9.8 kcal/mol (10.7 kcal/mol with 6-31+G*) with regards to the separated entities. When including the ZPE correction (3.2 kcal/mol) and BSSE (0.9 kcal/mol), this complexation energy decreased to 5.7 kcal/mol. In that configuration one of the sulfur atoms is equidistant from two NH_2 groups (Figure 6), located at 3.94 Å from C of $C(NH_2)_3^+$ with $\theta = 180^\circ$. When this distance increases, another secondary minimum (at $\theta = 0^\circ$) was obtained with an energy of 4 kcal/mol above the minimum. This configuration is very close to that in lysozyme, one S atom is now near only one NH_2 group (**Figure** 6). Indeed the proximity of S atoms and amine groups has been observed in many proteins.[52,53]

ii. Radical Complexes. We have considered now addition of an electron to the stable cationic complexes and studied various radical complexes with the same strategy as that used for the cationic complex; that is, as a first approximation, a scan was performed varying d and θ characterized in Figure 5, the other intra- and intermolecular geometrical data being fixed. We observed two possibilities of electronic localization either on the disulfide molecule or on the guanidinium ion:

$$[C(NH_2)_3^+\cdots S_2H_2] + e \rightarrow [C(NH_2)_3^+ ... S_2\ H_2^{\bullet -}] \quad (1)$$

$$[C(NH_2)_3^+\cdots S_2H_2] + e \rightarrow [C(NH_2)_3^{\bullet}\cdots S_2H_2] \quad (2)$$

So we obtained two different types of complexes, zwitterionic (1) and neutral (2); each of them represents the solution corresponding to the lower state at the MP2 level calculations. The minimum energy configurations were then fully optimized. (Tables 6 and 7). It can be noticed that the most stable configurations are zwitterionic.

Zwitterionic Complexes $C(NH_2)_3^+\cdots S_2H_2^{\bullet -}$. ZW1 corresponds to the most stable cationic complex with θ close to 180° (Figure 7a). The sulfur closest to the guanidinium ion bears a net charge of $-0.77e$ and strongly interacts with H atoms of the NH_2 group at a short distance of 2.20 Å. The other sulfur has a charge of $-0.21e$. The intramolecular parameters of each entity, disulfide and guanidinium ion, in the complex are close to those of isolated compounds: the SS bond has a 2.83 Å length, as in $H_2S_2^{\bullet -}$ and $C(NH_2)_3^+$ remains almost planar.

ZW2 is related to the second stable cationic complex near the lysozyme configuration and is close to $\theta = 0^\circ$. Conversely to the situation of the cationic complexes, this configuration is the most stable. Therefore the relative energy E_r of all other

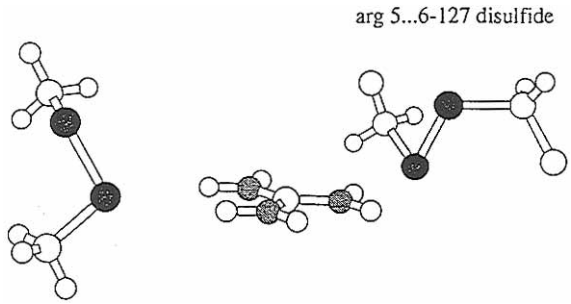

Figure 6. Minimal and experimental configurations of the cationic complex. The secondary minimum corresponds to the experimental configuration of disulfide bridge 6−127 and arginine 5 in lyzozyme, as noted in the figure. S atoms are represented in black and N in gray.

TABLE 6: Structures and Energies of the Stable Zwitterionic Complexes, $C(NH_2)_3^+\cdots S_2H_2^-$, at Different Levels of Correlation with the 6-31G* and 6-31+G* Basis Sets: All the Shortest Intermolecular Distances between atoms C and H of $C(NH_2)_3$ and S of H_2S_2 ($d_{C\cdots S}$ and $d_{H\cdots S}$) Are Given in Angstroms, and Total Energies Are in Atomic Units; In Parentheses, Relative Energies E_r Are in kcal/mol; Q_{S_1} and Q_{S_2} are the Sulfur Atomic Net Charges

	ZW1	ZW2	ZW3	ZW4
		6-31G*		
$d_{C\cdots S}$	3.566	3.814	2.841	3.511
$d_{H\cdots S}$	2.197	2.184	3.228	2.620
E_{PMP2}	−1001.68761	−1001.70163	−1001.68267	−1001.67470
E_r	(8.8)	(0.0)	(11.9)	(16.9)
E_{PMP3}	−1001.74040	−1001.75368	−1001.73527	−1001.72681
E_r	(8.3)	(0.0)	(11.6)	(16.9)
$E_{MP4SDTQ}$	−1001.77553	−1001.78895	−1001.77130	−1001.76356
E_r	(8.4)	(0.0)	(11.1)	(15.9)
Q_{S_1}	−0.77	−0.60	−0.77	−0.67
Q_{S_2}	−0.21	−0.43	−0.30	−0.27
		6−31+G*		
$d_{C\cdots S}$	3.568	3.929	2.841	3.505[a]
$d_{H\cdots S}$	2.201	2.165[b]	3.228	2.567
E_{PMP2}	−1001.71094	−1001.72450	−1001.70955	−1001.70212
E_r	(8.5)	(0.0)	(9.4)	(14.0)

TABLE 7: Structures and Energies of the Stable Neutral Complexes, $C(NH_2)_3\cdots S_2H_2$, at Different Levels of Correlation with the 6-31G* and 6-31+G* Basis Sets: All the Shortest Intermolecular Distances between Atoms C of $C(NH_2)_3$ and S of H_2S_2 ($d_{C\cdots S}$) are given in Angstroms and Total Energies Are in Atomic Units; In Parentheses, Relative Energies E_r Are in kcal/mol; Q_{S_1} and Q_{S_2} Are the Sulfur Atomic Net Charges

	6-31G*		6−31+G*	
	N1	N4	N1	N4
$d_{C\cdots S}$	4.477	4.451	4.077	4.416
E_{PMP2}	−1001.64651	−1001.65951	−1001.67752	−1001.68786
E_r	(34.6)	(26.4)	(29.5)	(23.0)
E_{PMP3}	−1001.70505	−1001.71713		
E_r	(30.5)	(22.9)		
$E_{MP4SDTQ}$	−1001.73813	−1001.75046		
E_r	(31.9)	(24.2		
Q_{S_1}	−0.14	−0.15		
Q_{S_2}	−0.09	−0.15		

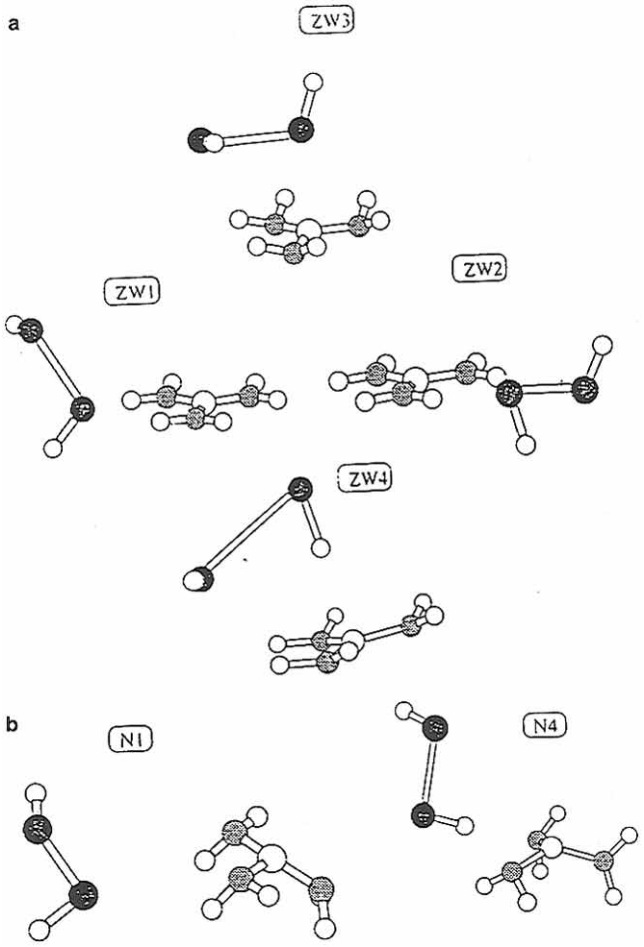

Figure 7. Optimized configurations of (a) the zwitterionic radical and (b) the neutral radical complexes.

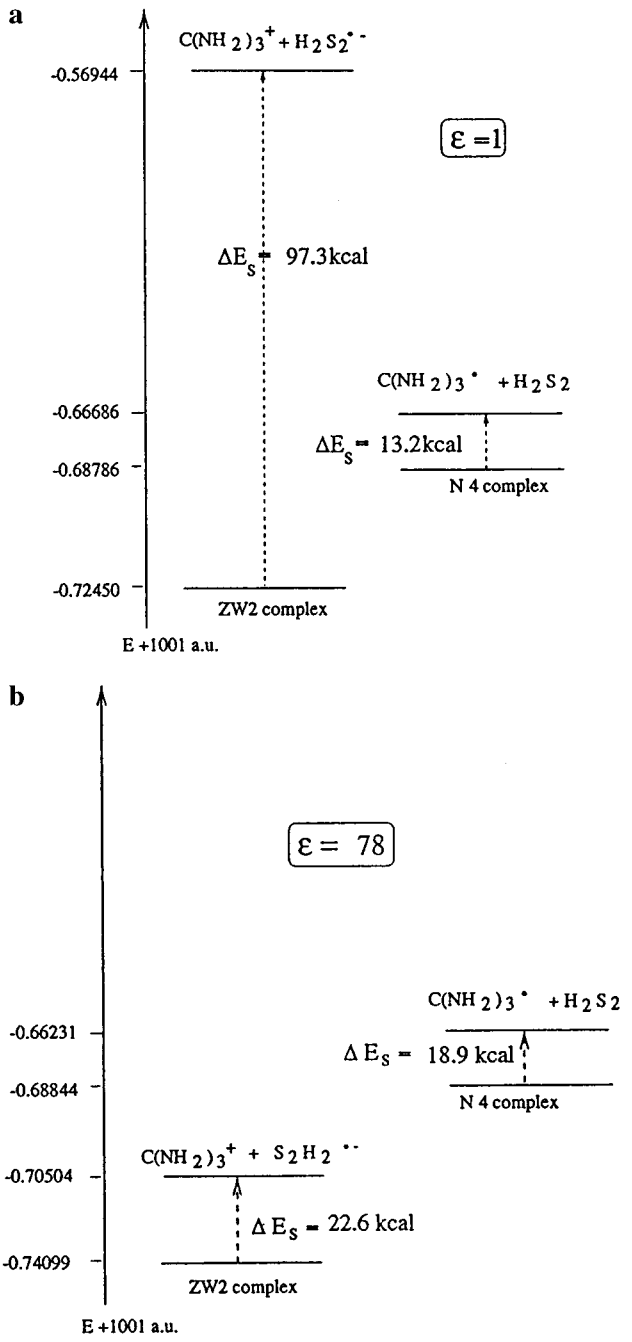

Figure 8. Diagrams of the energy levels of the most stable zwitterionic (ZW2) and neutral (N4) radical complexes and their separated entities in different media within MP2/6−31+G* calculations. Complexation energies, ΔE_s, are indicated on dashed arrows: (a) in vacuum ($\epsilon = 1$); (b) in water ($\epsilon = 78$).

complexes is defined with regards to ZW2 (Tables 4, 6, and 7). The ZW1 energy is then 8.8 kcal/mol higher than that of ZW2 (Table 6). An analysis of Mulliken populations has pointed out different electron distributions of negative charge between the two sulfur atoms between ZW1 and ZW2 complexes; in the latter the disulfide bond is in the guanidinium plane, the two sulfur atoms being equally close to the NH_2 groups, and the negative charges are more equally distributed between them (Table 6).

The third zwitterionic complex, ZW3, is close to $\theta = 90^\circ$ and is a particular configuration in which one sulfur is above the vacant orbital of the carbon of the guanidinium ion. It has a quite higher energy (E_r =11.9 kcal/mol, Table 6) and is in equilibrium with another type of configuration (ZW4) resulting from the overturning of hydrogen around the SS bond. This last configuration, where the hydrogen of the disulfide is pointing toward the carbon atom (Figure 7a, Table 6), has an even higher energy (16.9 kcal/mol) but remains stable.

Neutral Complexes $C(NH_2)_3\bullet\cdots S_2H_2$. The neutral complex (N1) (Figure 7b) is in a configuration close to that of ZW1 with $\theta \simeq 180^\circ$ but with a higher energy (E_r = 34.6 kcal/mol, Table 7). H_2S_2 is globally neutral, with charges of ca. 0.1*e* on sulfur. The electron excess is mostly on the carbon atom of the guanidinium radical (0.56*e* instead of 0.91*e*). As for the zwitterionic complex, the intramolecular geometry parameters are not changed by the complexation; the SS bond length remains equal to 2.05 Å and $C(NH_2)_3\bullet$ is pyramidal.

We also obtained a neutral complex (N4) related to the zwitterionic ZW4 with a higher energy (E_r = 26.4 kcal/mol, Table 7, Figure 7b). All complexes were also optimized with the 6−31+G* basis set at the MP2 level. The geometrical parameters as well as the energy variations are little modified (Tables 6 and 7); the zwitterionic ones, especially ZW2, are always the most stable. Single-point calculations at MP4SDTQ/6-31G* were carried out on these configurations (Tables 6 and 7) and confirm the stabilities of these complexes and their relative order. In Figure 8, we have summarized an energetical diagram with the levels of the most stable isolated species before and after one-electron reduction and the most stable ZW2 and N4 radical complexes. The complexation energy, ΔE_s, is roughly estimated by $\Delta E_s = E_{complex} - (E_{sulfur} + E_{guanidinium})$.

In Figure 8 BSSE and ZPE corrections are not included in these results, but they remain rather small; for instance BSSE varies from 1 kcal/mol (for neutral complexes) to 3 kcal/mol (for zwitterionic ones). These small values of BSSE for complexes are not very surprising when 6-31G* and 6-31+G* basis sets are used, as was also pointed out for the base-pairing energies in DNA.[43a] ZPE corrections are sligthly higher but remain lower than 5.5 kcal/mol (maximum value obtained for the ZW2 complex). Furthermore we noticed that the ZPE corrections are proportionally less important for BDE and complexation energies than for electronic affinities of the isolated sulfur species. So we may conclude that all these complexes are in stable configurations, especially ZW2, which is found to have substantially greater complexation energies than the neutral radical parents. Since this energy is mainly a result of interactions between S and NH_2 groups, increased charge on the sulfur contributes to strengthen it. Environment effects were estimated for these most stable radical complexes, ZW2 and N4, with both extreme dielectric constants, $\epsilon = 78$ and $\epsilon = 2$, as was described above for isolated species. These complexes being globally neutral, the corrections due to the Born charge terms are not to be taken into account. The values of energies in various media are summarized in Table 4. Comparison of these energies to those obtained in vacuum (Table 4, Figure 8) points out the differences between both radical complexes immersed in various media. As expected, ZW2 is more stabilized by solvation in a polar medium than by nonpolar medium, whereas N4 is always slightly stabilized. Nevertheless ZW2 is always favored with regards to N4, their relative energies varying from 25.2 kcal/mol ($\epsilon = 2$) to 36.0 kcal/mol ($\epsilon = 78$). Figure 8b shows that the complexation energies, ΔE_s, for ZW2 and N4 in water ($\epsilon = 78$) became of the same magnitude, slightly larger for ZW2, conversely to the situation in vacuum ($\epsilon = 1$, Figure 8a).

Conclusion

The reinvestigation of the one-electron reduction of lysozyme brought new elements: first we demonstrate that the reduction is highly specific to only one disulfide bridge, the 6−127 one. We may therefore postulate that the inital site of attack of $CO_2^{\bullet-}$ is this bond. Examination of the three-dimensional structure of the protein in the Protein Data Bank shows that indeed it is the SS bond most exposed to the solvent, which would be in agreement with the kinetic specificity. To our knowledge this is the first time that such a specificity is demonstrated for radiolytic reduction. Second, the reaction leads to two types of products: thiol functions coming from SS bond rupture, and fragmentation. The specificity of attack of $CO_2^{\bullet-}$ to disulfide is known.[3] Thus this fragmentation which is observed only with lysozyme has to be initiated by electron migration from the disulfide anion along the polypeptide chain. The theoretical investigation points out the following facts: the stability of the disulfide radical anion alone is indicated by our calculations, in vacuum as well as in a solvent, in agreement with pulse radiolysis experiments.[7−13,29,30] The guanidinium group and, more precisely, the amine groups increase the stability of the disulfide radical anion by electrostatic interactions. Although, in vacuum and for a dielectric constant of 2, the guanidinium ion has the most favorable EA values, electron addition to disulfide−arginine cationic structure occurs more favorably on disulfide, independently of the dielectric constant. Nevertheless, two types of radical complexes are obtained: neutral and zwitterionic. As in the NaCl case,[54] the zwitterionic complex is more stable. Both complexes differ by electron localization and subsequent geometrical transformations. It is interesting to note that the most stable radical complex is ZW2, whose geometry is the closest to the initial one in lysozyme. We can thus suggest that the specific reactivity of the 6−127 SS bond is also explained by the fact that the electron addition needs a very low reorganization energy, hence has a relatively low activation energy according to Marcus theory.[55] Other geometries could be attained by movements of the protein backbone, leading to the other stable complexes we found, but these movements would require energy. The electron localization seems very sensitive to variations of geometry as well as to the dielectric constant of the medium in which the disulfide is immersed. Nevertheless trapping of the electron by disulfides might occur whatever the environment. Geometrical parameter changes could be envisaged easily in the protein. This might lead to electron localization on arginine and end up by protein fragmentation.

Acknowledgment. We are indebted to Dr. V. Favaudon for numerous fructuous discussions and his participation in NMR experiments. The authors express thanks to Dr. J. Langlet for careful proofreading and critical comments on the manuscript. They thank the Institut du Développement et des Ressources en Informatique Scientifique (IDRIS) for providing computer facilities and technical assistance on Project 960268.

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