Experimental and Simulative Dissociation of Dimeric Cu,Zn Superoxide Dismutase Doubly Mutated at the Intersubunit Surface

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ABSTRACT The equilibrium properties of dimeric *Photobacterium leiognathi* Cu,Zn superoxide dismutase mutant bearing two negative charges in the amino acid clusters at the association interface has been studied, experimentally and computationally, and compared to those of the native enzyme. Pressure-dependent dissociation is observed for the mutant, as observed by the fluorescence shift of the unique tryptophan residue located at the intersubunit surface. The spectral shift occurs slowly, reaching a plateau after 15–20 min, and is fully reversible. Measurement of the degree of dissociation allows us to calculate the standard volume variation upon association and the dissociation constant at atmospheric pressure. On the other hand the native protein is undissociable at any pressure. In the simulative approach, the dissociation free energy has been calculated through the blue moon calculation method for the case of a multidimensional reaction coordinate, corrected for the rotational contribution within the semiclassical approximation for a free rigid-body rotor. The scheme permits to define a definite path for the rupture of the dimer and to calculate the effective force involved in the process. The calculated free energy difference is close to the experimental one, and the value obtained for the mutant is well below that obtained for the native protein, indicating that the theoretical reaction scheme is able to reproduce the experimental trend. Moreover, we find that, when the separation distance increases, the protein structure of the monomer is stable in line with the fast recovery of the original fluorescence properties after decompression, which excludes the presence of partly unfolded intermediates during the dimer-monomer transition.

INTRODUCTION

Noncovalent interactions between individually folded proteins, usually referred to as protein-protein interactions, drive fundamental phenomena in biology such as the formation of hormone-receptor, antibody-antigen, or enzyme-substrate complexes. Moreover, they are responsible for the formation and stability of quaternary structure in proteins. The ability of two or more molecules to bind selectively to each other forming noncovalent complexes is defined as molecular recognition. In this field, protein-protein recognition in water plays a particular role, due to the large molecular surfaces that are desolvated during the association. The physical-chemical basis of the process is clear from several studies (Janin, 1995): direct interactions between the ligands and the entropy gain of water removed from the contact interface provide the attractive contribution to the free energy of binding that more than compensates for the loss of translational and rotational entropy in the formation of the stable complex. Hence, the role of the contact interface is crucial. Several protein-protein interfaces has been extensively characterized at atomistic level (Lo Conte et al., 1999; Chakrabarti and Janin, 2002). Even if the proteins implicated in these processes are extremely diverse, their recognition sites share common properties, being generally hydrophobic, but also having several polar residues, especially when the complex is formed by prefolded molecules.

The modulation of the quaternary structure of proteins has been widely studied as examples of molecular recognition, and several cases have been reported where mutation of interface residues or ligand or metal binding has brought to a perturbation of the oligomeric state (D'Orazio et al., 2000; Riley-Lovingshimer et al., 2002; Barrera et al., 2002; Piana et al., 2003; Khayat et al., 2004; Doucette et al., 2004). An interesting case of molecular recognition is represented by dimeric Cu,Zn superoxide dismutase (SOD). This class of enzyme is widely studied, and, in particular, Cu,Zn SOD from *Photobacterium leiognathi* bacterion, has been extensively characterized at a structural and functional level (Bordo et al., 1999, 2000). The three-dimensional structure of PSOD has indicated the presence of three distinct clusters of residues at the interface crucial for dimer assembly: the first is centered on the side chains of Trp-83 and Phe-81, including other residues such as Val-29 and Pro-106, the second on Met-41 and Phe-96, and the third is formed by the residues located at the top of the interface, Lys-25, Tyr-26, and Asp-85, which are engaged in a hydrogen-bonded network. In a previous study (Cioni et al., 2003), we have shown that the native dimeric enzyme cannot be dissociated even at high pressure, and that the monomeric species can only be obtained upon removal of the metal cofactors. In line with

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these results, a theoretical approach based on the blue moon ensemble method (Carter et al., 1989; Sprik and Ciccotti, 1998) provided a large value for the free energy associated with the dissociation process of the native holo protein (Sergi et al., 2002).

In recent years, the numerical calculation of free energy differences in biomolecular systems has been widely applied as an efficient tool to provide insight concerning the origin of experimental results (Simonson et al., 2002). The most popular approach involves thermodynamic integration over variables parametrizing the Hamiltonian of the system (Frenkel and Smit, 2002). As an alternative approach, constrained molecular dynamics (MD) methods and in particular the blue moon ensemble method (Carter et al., 1989; Sprik and Ciccotti, 1998), provides free energy differences through integration along a path defined by varying a collective variable of the system, usually called reaction coordinate.

In this work, to reduce the nonbonded interaction that stabilizes the dimer, both the Val-29 and the Met-41 interface residues (Fig. 1), that belong to two of the three clusters crucial for the dimer assembly (Bordo et al., 1999), have been mutated into two negatively charged glutamic acid residue (DM SOD), and the effect of the mutation has been followed through experimental and simulative dissociation. The simulative ΔG value, evaluated through the blue moon calculation method, corrected for the rotational contribution, well matches the experimental result indicating that the theoretical scheme is a suitable to evaluate the equilibrium properties of a complex bimolecular system.

MATERIALS AND METHODS

Protein mutation and purification

The double-site mutant Val-29Glu and Met-41Glu of the recombinant Cu,Zn PSOD were prepared in a two-step polymerase chain reaction approach, according to Landt et al. (1990). The amplified DNA was restricted with *Eco*RI and *Hin*dIII and subsequently cloned into vector pEMBL18, previously digested with the same restriction enzymes. The expression plasmid obtained was inserted into the *Escherichia coli* strain 71/18 as for wild-type PSOD. Recombinant clones were grown in standard LB medium containing AMP (70 mg/l) for 5 h at 37°C. Then 1 mM IPTG (Sigma, St. Louis, MO), 0.25 mM of CuSO4, and 80 μM of ZnSO4 were added, and the cells were left to grow overnight. Periplasmic extracts were prepared as previously described (Foti et al., 1997), concentrated by ultrafiltration and loaded onto a gel filtration fast performance liquid chromatography column previously equilibrated with 20 mM Tris-HCl, pH 7.0, 0.15 M NaCl, to separate monomeric and dimeric forms of the enzyme. Further purification of the two enzyme forms has been carried by ion exchange chromatography, accordingly to previously described procedures (Foti et al., 1997). Proteins were purified to 98%, as judged by SDS-PAGE electrophoresis. Protein concentration was determined by the Lowry method (Lowry et al., 1951). The copper content of the protein samples, determined by electron paramagnetic resonance spectroscopy using a Cu^{2+}-EDTA solution as a standard, indicated the presence of one copper atom for each subunit showing that the protein is fully metallated. The activity assay, carried out with the pyrogallol method (Marklund and Marklund, 1974), indicated that the mutated SOD has an activity identical to the wild type.

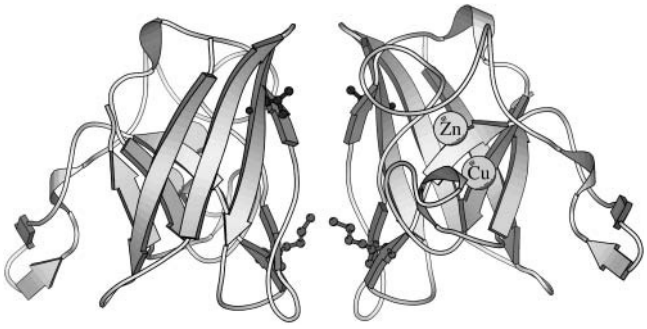

FIGURE 1 Interface region of the *Photobacterium leiognathi* Cu,Zn superoxide dismutase. The structure of the protein is indicated by the gray arrows, representing the β-strands, and by the thin wires, representing the random-coil structure and the turns. The spheres represent the copper and the zinc ions. The Val-29 and Met-41 residues mutated in Glu are represented as ball-and-stick models in dark and light gray, respectively. This picture has been obtained using the program Molscript (Kraulis, 1991).

Luminescence measurements

Fluorescence spectra, intensities were obtained with a home-made apparatus described previously (Cioni and Strambini, 1994). Briefly, continuous excitation for fluorescence spectra was provided by Cermax xenon lamp (LX 150 UV, ILC) and the excitation wavelength, typically 290 nm, was selected by a 0.25-m grating monochromator (model 82-410, Jarrel-Ash) with a 10-nm band pass. The emission was collected through another 0.25-m grating monochromator (Jobin-Yvon, H25, Lille, France) with 7-nm bandwidth detected by an EMI9635QB photomultiplier (Thorn EMI, Rockaway, NJ).

Luminescence measurements under pressure were carried out by placing the sample cuvette in a pressure cell (SITEC, Zurig), provided with sapphire windows and employing water as pressurizing fluid. Particular care was taken to assure temperature equilibration of the sample after each pressure variation, which required at least 5 min.

The degree of dissociation was determined from the displacement of the center of mass of the Trp fluorescence spectrum as described elsewhere (Ruan and Weber, 1989). Briefly, the center of spectral mass (ν_γ) is defined by the equation

$$\nu = (\Sigma \nu_i F_i)/(\Sigma F_l), \tag{1}$$

where F_1 is the fluorescence emitted at wavenumber ν_i.

The degree of dissociation (α) is related to ν_g by the following expression:

$$\alpha = [1 + Q(\nu_p - \nu_M)/(\nu_D - \nu_p)]^{-1}, \tag{2}$$

where Q is the ratio of the quantum yields of monomer and dimer, $(\nu_g)_p$ is the center of spectral mass at pressure p, and ν_M and ν_D are the corresponding quantities for monomer and dimer. The value of ν_D is calculated from the fluorescence spectrum at atmospheric pressure of the double mutant SOD. The value of ν_M refers to the central mass of the protein measured at 6 kbar.

Computational methods

Molecular dynamics simulation has been used to study a mutant of the dimeric protein superoxide dismutase from *Photobacterium leiognathi* in water. The coordinates of the wild-type *Photobacterium leiognathi* Cu,Zn superoxide dismutase at 2.1 Å resolution (Bordo et al., 1999) were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (Berman et al., 2000; web address http://www.rcsb.org/pdb; entry code

1BZO), and have been used to model the coordinates of the mutated enzyme. Both the residues Val-29 and Met-41 of the wild-type protein have been changed into a glutamic acid residue. After changing the side-chain atoms, these residues have been checked against contacts with the other residues. The side chains of the substituted residues easily accommodate in the dimer interface. Modeling was carried out by using the computer program Sybyl 6.0 by Tripos Associates (St. Louis, MO). For sake of clarity in this work the sequential numbering of the PSOD chain (from Gln-1 to Gln-151) applied by Bourne and collaborators (Bourne et al., 1996) has been used.

All simulations were performed with the DL-PROTEIN package (Melchionna and Cozzini, 2001). The subroutines performing effective force calculations explained before were introduced in the latest version of the code. We used the parameters set and partial charges of GROMOS-37c (Van Gunsteren and Berendsen, 1987). Few harmonic springs have been used to connect the atoms of the reaction coordinate to their neighbors in the proteins (see Results section). Within the united atoms approach of the force field adopted the nonpolar hydrogens of the protein are not explicitly taken into account, but represented by modifying the interaction parameters of the atoms to which they are linked. The molecule was put in a rectangular box filled with water solvent. An equilibrated SPC/E water (Berendsen et al., 1983) configuration was used as a building block, and water molecules whose atoms were less than 1.8 Å from any protein atom were removed. The final number of water molecules in the system was 10,344. All covalent bonds in the proteins and in water have been represented by constraints that keep fixed the bond distances to the proper chemical values. During the dynamics these bonds were kept fixed using the SHAKE algorithm (Ryckaert et al., 1977). In all simulations periodic boundary conditions were used (Frenkel and Smit, 2002). Van der Waals interactions were cut off at a distance of 10 Å, whereas electrostatic interactions were calculated by Ewald sums using the smooth particle mesh Ewald method (Esmann et al., 1995). The cutoff of the real space part was 10 Å, and the Ewald convergence parameter was set to 0.35. Sixth-order cubic splines were used for interpolation, with grids of 90·64·64 $Å^3$. Equations of motion were integrated with the Velocity Verlet scheme (Andersen, 1983) with a time step of 0.5 fs. The choice of such a small value was forced by the introduction of the harmonic springs in the protein model. The atoms defining the reaction coordinate (see Results section) are the C_α atoms of residues Gly-40, Val-107, Ala-121, and Asn-130. Coordinates of all the systems were saved every 100 fs for data analysis. A standard MD simulation of DM SOD was performed. The molecule was surrounded with 6824 SPC/E water (Berendsen et al., 1983) molecules. The GROMOS-37c force-field parameters have been used. For the SPME method the convergence parameter was set to 0.36 and sixth-order cubic splines were used for interpolation, with a grid of 64·64·64 $Å^3$. The simulation was performed in the NPT ensemble (Melchionna and Ciccotti, 1997) with $T = 300$ K and $P = 1$ atm, integrating the equations of motions with the Velocity Verlet scheme with a time step of 1 fs.

RESULTS

Experimental dissociation

The reversible process of monomerization of Cu,Zn SOD has been followed by fluorescence spectroscopy taking advantage of the presence of a single tryptophan residue, Trp-83, present in each subunit and located at the intersubunit interface. The fully metallated dimeric double mutant Met-41Glu/Val-29Glu is characterized, as the wild type, by a fluorescence spectrum having an emission maximum centered at 330 nm characteristic of an indole group partially screened from the solvent. Upon subunit dissociation Trp-83 becomes exposed to the solvent and its fluorescence maximum shifts to the red for an increased polarity of the chromophore's environment. Such a shift has been recently used to evaluate the role of metals in modulating the dimer to monomer dissociation process of wild-type SOD (Cioni et al., 2003).

Trp fluorescence spectra of fully metallated wild-type SOD is not altered by pressure up to 6.5 kbar. The only effect of pressurizing the holo protein is a modest 2-nm red shift of the spectrum, which is concentration independent and elastic, i.e., immediately recovered on decompression. Small spectral shifts are typically observed in monomeric proteins and are attributed to slight conformational changes/enhanced hydration induced by compression (Silva and Weber, 1993). On the other hand compression induces a large shift of the fluorescence spectrum of holo Met-41Glu/Val-29Glu mutant. Fig. 2 shows the fluorescence spectra at atmospheric pressure and at 6.5 kbar of 50 μM DM SOD. As often found with oligomer dissociation the spectral shift proceeds slowly and a plateau is reached only after 15–20 min, at 20°C. This was considered the minimum equilibration time and therefore all measurements were conducted after 30 min of pressurization. The red shift, calculated from the average emission energy (center of spectral mass ν_p, ($\nu_p = \Sigma\nu_i F_i/\Sigma F_i$)) measured in cm^{-1}, for the DM SOD plotted against pressure is reported in Fig. 3. The curve corresponds to a unimodal process that reaches a plateau at 6.5 kbar indicating that, at this pressure, the dissociation of dimers to monomers is complete. The spectral shift is fully reversible and the recovery of the fluorescence properties after decompression is immediate ($t <$ 4 min). The prompt recovery of the fluorescence properties on decompression and the lack of any hysteresis during the decompression part of the cycle (Fig. 3) are consistent with a relatively stable structure for the isolated subunit as already observed in the copper-depleted SOD (Cioni et al., 2003) and

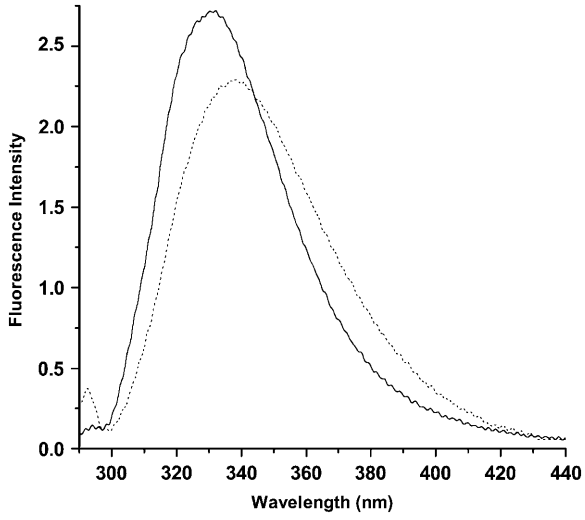

FIGURE 2 Effect of pressure on the intrinsic fluorescence emission of Met-41Glu/Val-29Glu SOD 50 μM in 0.01 M Tris HCl (pH 7.5). Intrinsic fluorescence spectra at atmospheric pressure (*solid line*) and at 6.5 kbar (*dashed line*). Excitation wavelength was 290 nm.

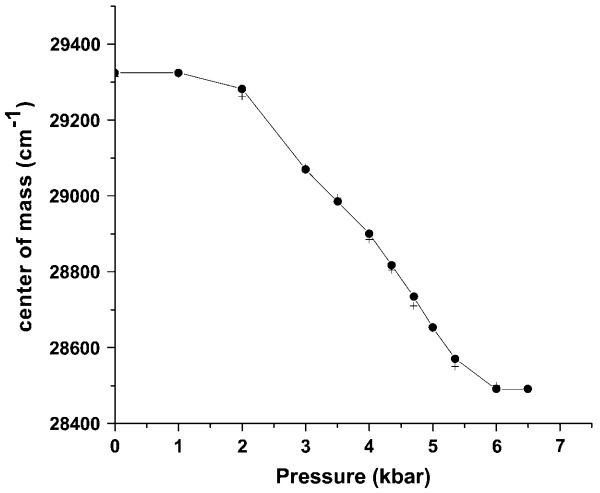

FIGURE 3 Center of mass of the intrinsic fluorescence of Met-41Glu/Val-29Glu SOD excited at 290 nm as a function of applied pressure. The value of the center of mass for each pressure is the average of five experiments. The data are for increasing (●) and decreasing (+) pressure. The protein concentration was 50 μM in buffer Tris HCl 0.01 M, pH 7.5.

at variance on what was observed during the monomerization process of other oligomeric proteins (Weber, 1992).

The degree of dissociation for DM SOD as a function of pressure, calculated from the change of the center of spectral mass ν_p is reported in Fig. 4, for protein concentrations of 5 and 50 μM. The dissociation profile is concentration dependent, the most dilute sample having the midpoint of the dissociation curve shifted toward lower pressure values. Such a result is an unambiguous evidence that permits us to

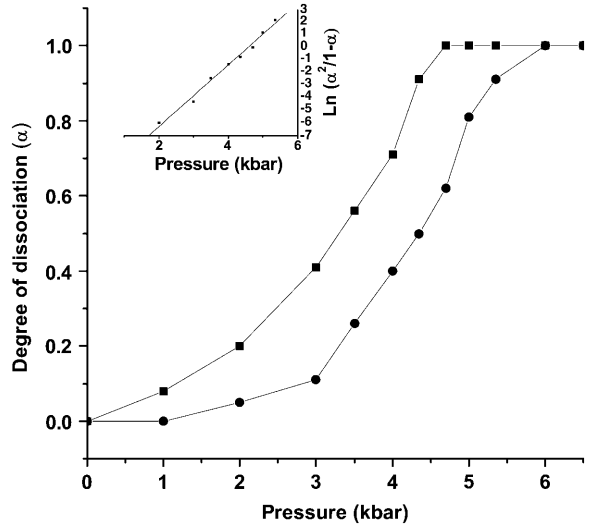

FIGURE 4 Effect of Met-41Glu/Val-29Glu SOD concentration on pressure dissociation: (■) 5 μM SOD; (●) 50 μM SOD. Degrees of dissociation were calculated from measurements of center of spectral mass of intrinsic fluorescence (see Materials and Methods). The excitation was 290 nm. Other conditions were the same as in Fig. 2. (*Inset*) Plot of ln (α^2/1 − α) versus pressure for 5 μM protein.

attribute the spectral shift to the dimer-monomer dissociation process.

The standard volume change upon dissociation (ΔV) and the dissociation constant at atmospheric pressure (K_{d0}) can be estimated from pressure-dissociation data using the following thermodynamic relation

$$K_{d(p)} = K_{d0} \exp(-p\Delta V/RT), \quad (3)$$

where $K_{d(p)}$ is the dissociation constant at pressure p. Introducing α_p, the degree of dissociation at pressure p, Eq. 3 assumes the following form for the case of a dimer-monomer equilibrium:

$$ln(\alpha_p^2/(1-\alpha_p)) = p(\Delta V_p/RT) + ln(K_{d0}/4C_0), \quad (4)$$

where C_0 is the molar protein concentration expressed as dimer.

The inset in Fig. 4 shows the plot of $\ln(\alpha_p^2/(1-\alpha_p))$ versus pressure for DM SOD at 5 μM. The association volume change (ΔV_p) derived from the slope is 60 ± 5 mL/mol and the dissociation constant at atmospheric pressure (K_{d0}) obtained from the extrapolation to the ordinate axis is $2.5 \times 10^{-9}\text{M}^{-1}$ that corresponds to a free energy of dissociation (ΔG_0) of 11.5 Kcal/mol. The volume change upon dissociation can also be determined from the shift of the midpoint pressure ($P_{1/2}$) measured on the pressure-dissociation curve at two different protein concentration. For monomer-dimer equilibrium, the difference in the midpoint of the pressure dissociation curve ($dP_{1/2}$, at which 50% dissociation is observed) is related to the volume change ΔV_c and the protein concentration C_2 and C_1 by the following expression (Weber, 1992)

$$dP_{1/2} = RT/\Delta V_C \; ln\, C_2/C_1. \quad (5)$$

The value of ΔV_C obtained from the difference in $P_{1/2}$ observed at a protein concentration of 5 and 50 μM is 58 ± 3 mL/mol, identical within the experimental error to the ΔV_p value calculated from Eq. 3. The agreement between ΔV_p and ΔV_C suggests that the dissociation process has a stochastic character, unlike the pressure dissociation of large protein aggregates (Weber, 1992; Erijman and Weber, 1991). Moreover this value is much larger than that obtained from the dissociation curve of copper-depleted wild-type SOD so explaining the different propensity in dissociation of native and DM SOD.

Simulative dissociation

Constrained molecular dynamics dissociation

Information at an atomistic level on the dissociation process of DM SOD can be obtained by MD simulations. Recently, we have developed a free energy calculations scheme based on a constrained MD technique and applied it to evaluate the dissociation equilibrium of wild-type SOD (Sergi et al., 2002). This technique is a generalization of the blue moon

ensemble method (Carter et al., 1989, Sprik and Ciccotti, 1998) for the case of a multidimensional reaction coordinate. The rupture process of the dimeric protein is evaluated choosing a specific separation path keeping fixed the relative monomer orientation and calculating the effective force involved in the process. The separation could be fully described by constraining the relative distance as a scalar reaction coordinate and sampling all the other degrees of freedom of the system, including the slow rotational motion of each monomer. However, the sampling of the overall orientational motion of the monomers would require very long trajectories, due to the size of the molecules. Hence, we produce a reaction path that separates the monomers at a fixed monomer orientation. This means that we calculate the free energy of the association process at a given orientation.

To describe the separation of the monomers in terms of their relative distance and orientation, we have to define a vectorial reaction coordinate. This can be done using a set of four atoms in each monomer, which define a roughly rigid frame in each subunit, permitting us to identify a six-dimensional reaction coordinate

$$(\xi_1, \xi_2, \xi_3, \xi_4, \xi_5, \xi_6) = (D_x, D_y, D_z, \theta, \phi, \psi), \tag{6}$$

where D_x, D_y, and D_z are the components of the distance vector between the centers of mass of two properly chosen sets of atoms, and θ, ϕ, and ψ are the Euler's angles between two frames attached on each monomer, and constructed using the same sets of atoms as previously described in detail elsewhere (Sergi et al., 2002; Maragliano et al., 2004). As an illustration, in Fig. 5 the positions of the two sets of atoms in the protein are shown, together with their centers of mass. Providing that there is no mixing between intramolecular and reaction coordinate constraints (Coluzza et al., 2003), the effective force associated to the ξ_α component of the reaction coordinate is calculated as

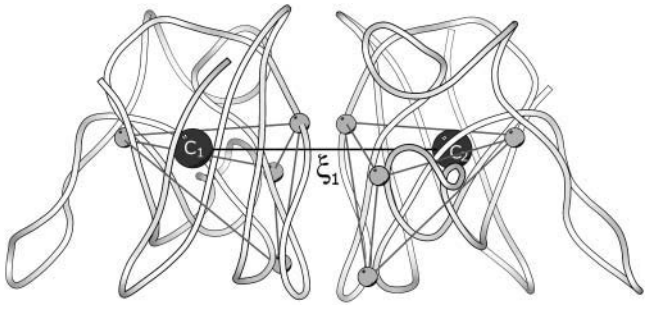

FIGURE 5 Thin wire representation of the PSOD backbone scaffold. The C_α atoms of residues Gly-40, Val-107, Ala-121, and Asn-130, of each subunit, are represented as small light gray spheres. The two large dark gray spheres labeled C_1 and C_2 represent the centers of mass of the two sets of atoms. The *x*-component of the vector distance between C_1 and C_2, D_x, defines the first component of the reaction coordinate, ξ_1, whose different values are used to build the separation path. This picture has been obtained using the program Molscript (Kraulis, 1991).

$$F_{\xi_\alpha} = \frac{\langle |\Xi|^{-1/2} \left[\frac{k_B T}{2} G_\alpha - \lambda_\alpha \right] \rangle_{\xi'}}{\langle |\Xi|^{-1/2} \rangle_{\xi'}}, \tag{7}$$

where the symbol $\langle \cdots \rangle_{\xi'}$ denotes equilibrium averages over a trajectory with the constrain $\xi = \xi'$, λ_α is the Lagrange multiplier associated to the constraint on ξ_α,

$$G_\alpha = \sum_{i,j} \frac{2}{m_i m_j} \sum_{\mu\sigma\nu} \Xi^{-1}_{\mu\alpha} \frac{\partial \xi_\mu}{\partial \mathbf{r}_i} \frac{\partial^2 \xi_\sigma}{\partial \mathbf{r}_i \partial \mathbf{r}_j} \frac{\partial \xi_\nu}{\partial \mathbf{r}_j} \Xi^{-1}_{\sigma\nu}, \tag{8}$$

and

$$\Xi_{\alpha\beta} = \sum_k \frac{1}{m_k} \frac{\partial \xi_\alpha}{\partial \mathbf{r}_k} \frac{\partial \xi_\beta}{\partial \mathbf{r}_k}. \tag{9}$$

The above-mentioned condition of no mixing between the different kinds of constraints is guaranteed by using in the protein model harmonic springs to represent the molecular connectivity of the atoms defining the reaction coordinate.

Given a path $\mathcal{C}(\xi^a, \xi^b)$ between two states identified by two different values of the reaction coordinate, the free energy variation when going from one state to the other is calculated as a potential of mean force

$$W(\xi_1^b, \ldots, \xi_n^b) - W(\xi_1^a, \ldots, \xi_n^a) = -\int_{\mathcal{C}(\xi_a, \xi_b)} \sum_{\alpha=1}^{n} d\xi'_\alpha F_{\xi'_\alpha}. \tag{10}$$

To get rid of the rotational degrees of freedom of the system, the ξ_4, ξ_5, ξ_6 components have been constrained to fixed values. Moreover, ξ_2, ξ_3 also have been constrained to consider a path described by different values of ξ_1 only. The initial value of the reaction coordinate, $\{\xi_1^0, \xi_2^0, \xi_3^0, \xi_4^0, \xi_5^0, \xi_6^0\}$, corresponds to a minimal energy configuration obtained after having first equilibrated the protein structure in water, and then quenched the system at zero temperature. The values $\{\xi_1^{(i)}, \xi_2^0, \xi_3^0, \xi_4^0, \xi_5^0, \xi_6^0\}$, identifying the other points of the separation path, have been obtained by separating the monomers in water as rigid bodies. In more details, starting from the initial coordinates $\{\xi_1^0, \xi_2^0, \xi_3^0, \xi_4^0, \xi_5^0, \xi_6^0\}$, we freeze the motion of the protein atoms by constraining all atomic positions, and assign a group relative velocity of 10^{-3} Å/fs along the direction defined by ξ_1, while integrating the motion of water molecules in an NVT ensemble (Nosè, 1984). This procedure allows water to diffuse following the monomers translation while avoiding undesired overlaps that could disrupt the protein structure, and to fill up smoothly the region at the interface. Once a distance increment of 1 Å is obtained from a configuration labeled with the value of the reaction coordinate $\{\xi_1^{(i)}, \xi_2^0, \xi_3^0, \xi_4^0, \xi_5^0, \xi_6^0\}$, the configuration with the corresponding $\{\xi_1^{(i+1)}, \xi_2^0, \xi_3^0, \xi_4^0, \xi_5^0, \xi_6^0\}$ is recorded. Following this procedure we produced 11 configurations of the monomers at various relative distances along ξ_1 and fixed relative orientation. The values of ξ_1 obtained

for the DM SOD are $\xi_1 = 16.78$, 17.78, 18.78, 19.78, 20.78, 21.78, 22.78, 23.78, 24.78, 25.78, 26.78 Å. Starting from such configurations, we fully equilibrate the system first by keeping protein atoms frozen and running water NVT dynamics at a constant temperature of 500 K for a few tens of picoseconds, to properly hydrate the interface, and finally at 300 K allowing full protein motion in the presence of the blue moon constraints on the values of the reaction coordinate $\{\xi_1^{(i)}, \xi_2^0, \xi_3^0, \xi_4^0, \xi_5^0, \xi_6^0\}$. After ~100 ps of this equilibration, we start the calculation of the effective force for each of the constraints values $\{\xi_1^{(i)}, \xi_2^0, \xi_3^0, \xi_4^0, \xi_5^0, \xi_6^0\}$ is started in the NVT ensemble with $T = 300$ K.

In Fig. 6 the results for the effective force and the potential of mean force for the separation path are reported. The barrier height for the separation process is 26.0 ± 1.6 Kcal/mol, a value relatively far from the experimental one, indicating that the used approach is a too-crude approximation to properly describe what is occurring. The discrepancy between experimental and calculated values can be rationalized considering that in our scheme the initial and final states maintain always the same relative orientation with a consequent negligence of the rotational degrees of freedom that then must be independently evaluated.

Calculation of the rotational contributions

When two separate molecules associate in a solvent, the unfavorable loss in the ideal contribution to the entropy related to translational and rotational degrees of freedom is recovered by favorable enthalpic (i.e., the internal energy of the associated molecule) and entropic contributions (from internal vibrational degrees of freedom and from the solvent because molecules at the binding surface gain motility; Tidor and Karplus, 1994). The calculation of free energy changes due to the loss of rotational and translational contribution in molecular association is a major issue in molecular biophysics and several approaches have been followed to correctly evaluate them (Swanson et al., 2004). In our procedure, described in the previous section, free energy differences are computed on a path where the separation of the monomers is performed by attaching a reference frame on each of them to constrain both the absolute and relative rotations along the path. Hence, we cannot compare directly the calculated free energy values with the experimental ones, without estimating the contributions due to the constrained degrees of freedom. To this purpose, we have used the method of Tidor and Karplus (1994), which, on the basis of a statistical mechanics framework developed by Chandler and Pratt (Chandler and Pratt, 1976; Chandler, 1979) for treating thermodynamic equilibria in solution, permits us to calculate the free energy of dissociation of a dimer in solution as a sum of gas-phase terms for the solute degrees of freedom and a term due to the interaction with the solvent. In principle, in the blue moon approach we have evaluated the contributions coming from the solute translational/vibrational degrees of freedom and from the interaction with the solvent, and hence the only missing term is the rotational contribution to the association free energy. This contribution has been evaluated for both the wild-type SOD and the DM SOD by applying the free rigid rotor approximation (Tidor and Karplus, 1994).

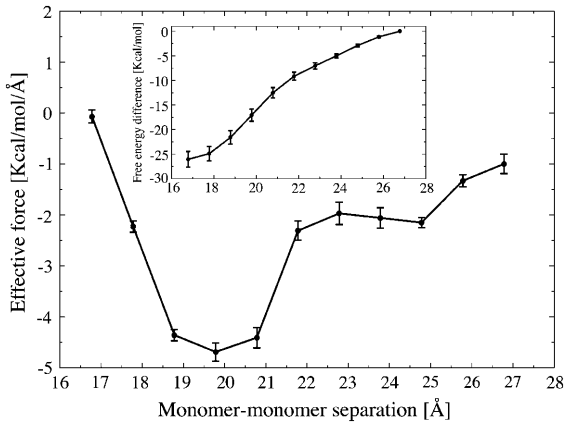

FIGURE 6 Separation path from a quenched protein configuration. The main graph shows the calculated mean force as a function of the monomer separation at given mutual orientation and relative position. The inset shows the associated free energy difference. The statistical errors (σ) are reported as indicated.

Under this approximation the rotational free energy corresponding to a rotating rigid molecule is given by McQuarrie (1976)

$$G_{\text{rot}} = -k_B T \left[\frac{1}{2} ln(\pi I_A I_B I_C) + \frac{3}{2} ln\left(\frac{8\pi^2 k_B T}{h^2}\right) - ln(\sigma) \right], \tag{11}$$

where, k_B is the Boltzmann constant, h is the Planck constant, T is the temperature, σ the symmetry factor, and $I_A I_B I_C$ are the principal moments of inertia.

The moments of inertia for the DM SOD have been computed using the average protein conformations calculated after 0.6- and 1.2-ns MD simulations. The relative difference is ~5% indicating that the macromolecules are fully stable during the trajectory. An identical calculation also has been carried out on the MD simulation of the WT SOD to include the rotational contribution to the free energy difference previously evaluated through the blue moon ensemble method (Sergi et al., 2002). The product of the three moments of inertia $I_A I_B I_C$ is 7.42×10^{18} and 4.48×10^{20} g^3A^6/mol^3 for the DM SOD monomer and dimer, and 9.57×10^{18} and 3.99×10^{20} g^3A^6/mol^3 for the WT SOD monomer and dimer, respectively. The rotational free energy contribution to the dissociation of the dimer calculated as $\Delta G_{\text{rot}} = 2G_{\text{rot}}(monomer) - G_{\text{rot}}(dimer)$ is summarized in Table 1, together with the net dissociation free energies obtained as a sum of the rotational and blue moon calculated contribution.

In the case of the DM SOD, the theoretical value matches closely the experimental result found using the

TABLE 1 Calculated and experimental ΔG values for the dimer-monomer dissociation of WT and DM SOD

	ΔG_{BM} (*kcal/mol*)*	ΔG_{rot} (*kcal/mol*)†	ΔG (*kcal/mol*)‡	ΔG_{exp} (*kcal/mol*)§
DM SOD	−26.01	14.73	−11.28	−11.50
WT SOD	−39.40	14.92	−24.48	–

*Blue moon calculation.
†Rotational contribution.
‡$\Delta G_{rot} + \Delta G_{BM}$.
§Experimental result; WT SOD is not dissociable even at high pressure.

pressure-dependent fluorescence spectroscopy, also reported in Table 1. The lack of an experimental ΔG value for the wild type make a direct comparison impossible, however, the 24.48 Kcal/mol theoretical value is in line with the non-dissociability of the protein at any pressure, being a value too large to be experimentally measured.

CONCLUSIONS

In this work the free energy difference related to the dissociation of doubly mutated dimeric Cu,Zn superoxide dismutase has been evaluated experimentally and through a simulative approach. Measurements of the fluorescence of the single tryptophan located at the dimer interface as a function of pressure allow us to unambiguously show that the introduction of two negatively charged residues at positions 29 and 41 in place of a valine and a methionine permit us to reversibly dissociate the protein. The corresponding ΔG value for DM SOD is 11.5 Kcal/mol, at variance with the dimeric native enzyme, which is undissociable even at high pressure (Cioni et al., 2003). In the simulative approach the dissociation free energy has been obtained through the blue moon free energy difference calculation method applied to the case of a specific path with fixed molecular orientation in a multidimensional reaction coordinate space, corrected to include the entropic effects of rotations. Rotational contributions were estimated in the semiclassical approximation for the free rotor considering both monomers and dimers as rigid molecules (Tidor and Karplus, 1994).

The sum of the two contributions quantitatively reproduces the experimental free energy value evaluated for dimer dissociation, indicating that the applied theoretical approach well approximates the dissociation phenomenon. The same procedure applied to the native enzyme gives a ΔG value of 24.48 Kcal/mol, a value that is still too high to be measured by the experimental dissociation under pressure but it is more close to the ΔG values estimated for many bimolecular systems (Janin, 1996), and then more reliable than the uncorrected theoretical value of 39 Kcal/mol previously quoted (Sergi et al., 2002).

The results reported here indicate that a careful application of the blue moon ensemble can provide a reliable strategy for the evaluation of the equilibrium properties of complex biomolecular systems such as a dimeric protein.

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