

Mixed Quantum-Classical Methods for Molecular Simulations of Biochemical Reactions With Microwave Fields: The Case Study of Myoglobin

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Abstract—Contradictory data in the huge literature on microwaves bio-effects may result from a poor understanding of the mechanisms of interaction between microwaves and biological systems. Molecular simulations of biochemical processes seem to be a promising tool to comprehend microwave induced bio-effects. Molecular simulations of classical and quantum events involved in relevant biochemical processes enable to follow the dynamic evolution of a biochemical reaction in the presence of microwave fields. In this paper, the action of a microwave signal (1 GHz) on the covalent binding process of a ligand (carbon monoxide) to a protein (myoglobin) has been studied. Our results indicate that microwave fields, with intensities much below the atomic/molecular electric interactions, cannot affect such biochemical process.

Index Terms—Bioelectromagnetics, microwaves bio-effects, molecular simulations, myoglobin.

I. INTRODUCTION

THE LAST two decades have seen a dramatic increase of biological experiments investigating possible effects of exposure to microwave electromagnetic (EM) fields, especially those emitted from mobile telecommunication devices. Such research has given rise to a huge literature on effects, both on animals (*in vivo*) and on cells or cell cultures (*in vitro*), yielding contradictory and often intriguing results [1], [2]. Regarding a well-studied tissue such as blood, many studies failed to find RF-induced effects, while others had success. Studies have been performed to evaluate the possible induction of genotoxic effects since they are of vital interest in the risk assessment on human exposure. There is a close correlation between DNA

damage and carcinogenesis. Two recent reviews on cytogenetic effects of RF fields indicate that scientific data remain too controversial to definitively establish or exclude an effect related to genetic damage [3], [4]. In order to overcome this uncertainty, more recent studies have investigated long-term *in vivo* activities [5], and there have been a number of large *in vitro* inter-laboratories coordinated projects, which gave rise to a large debate [6], concluded in no positive independent replications [7].

In studies of other biological end-points, Black and Heynick [8] reported that white cells (leukocytes) seem to be more sensitive than red cells (erythrocytes) to RF fields, but the effects on white cells were consistent with normal physiological responses to temperature fluctuations. Moreover, in studies investigating stress indicators, no effects have been reported for heat shock protein expression on lymphocytes [9], while for erythrocytes, a change in the oxidative stress was observed [10].

A possible key point to explain this lack of consistency is that, in most of the studies, the mechanisms for the RF effects were not investigated [8]. To initiate or promote effects in biological systems, EM fields must give rise to a series of events that ultimately lead to some outcome [11]. This chain of events starts with a field interaction with biological molecules or structures, altering their charge distribution, chemical state, or energy. This can be considered as the first “transduction” step for an effect to occur in a biological system, as schematically explained in [12, Fig. 1]. The change, at the molecular level, can be sensed and amplified through the biological scale of complexity to produce responses that might have consequences for the organism [13, Fig. 1].

Among the possible molecular processes to investigate, here we adopted one of the best established hypotheses of microwave effects on molecular systems, where, at the basis of protein function, a direct action of an exogenous field on the process of ligand binding is postulated [14]. The binding of a ligand to a protein receptor site is a chemical reaction where a substance is able to bind and form a complex with a biomolecule to serve a biological purpose.

Therefore, in this paper, the attention is focused on the covalent binding of carbon monoxide to myoglobin protein in pure water in the presence of a microwave EM field.

We consider myoglobin because it plays, as hemoglobin in blood tissue, an essential role in stabilizing molecular oxygen for transport and storage processes, through reversible binding of diatomic ligands as carbon monoxide, nitrogen monoxide, and diatomic oxygen. Recently it has been suggested that the

Manuscript received January 03, 2008; revised July 10, 2008. First published October 24, 2008; current version published November 07, 2008.

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Digital Object Identifier 10.1109/TMTT.2008.2005890

physiological role of myoglobin may be multifaceted, involving more than simple storage and transport of oxygen. Specifically, myoglobin may mediate oxydative phosphorylation, protect against oxydative damage, and inactivate enzymes [15]. Moreover, given its relatively small size and involvement in many relevant biological processes, it served as a prototype or a reference for the globin superfamily [16], and it has been considered as a prototype for protein physics and chemistry [17].

For these reasons, myoglobin is perhaps the most studied protein both experimentally and theoretically [18]; and we considered it the best molecular model in approaching the controversial problem of a direct action of microwave on chemical reactions. In a recent paper of Perreux and Loupy [19], an attempt to rationalize microwave effects in chemical reactions involved in organic synthesis is proposed. Despite the numerous experimental studies, it is claimed that more computational calculations and development of theories are still required [20], especially when dealing with reactions typical of protein complexes.

The theoretical approach proposed here is based on fully atomistic molecular simulations. Atomistic molecular simulations are a form of computer calculations in which atoms and molecules of a given system are considered to interact for a period of time under the known laws of physics, giving a view of the motion of the atoms. Since molecular systems generally consist of a vast number of particles, it is impossible to find the properties of such complex systems analytically. Therefore molecular simulations circumvent this problem by using numerical methods. They represent an interface between laboratory experiments and theory, and can be considered as “virtual experiments” [21].

In order to use this approach in studying the above-mentioned unsolved questions, it is necessary to consider the presence of the EM field. Recently, we have studied, in vacuum, the action of an EM field on the reaction of carbon monoxide binding to the isolated myoglobin receptor site, finding that only electric field intensities of the order of magnitude of 100 MV/m are able to induce significant variations [22].

The aim of this work is to study the carbon monoxide binding to myoglobin, considering the whole protein in water, under the exposure to a 1-GHz EM field, to understand if microwave fields of intensities lower than 100 MV/m could alter the binding or unbinding processes. We used a mixed quantum-classical method, the *perturbed matrix method* [23]–[25]. It has been used for solvated carbon monoxide providing thermodynamic properties of the solute with experimental validation of the data [26, Table III]. Successively, this mixed method has been used to investigate the carbon monoxide binding reaction in myoglobin solvated in water, again with experimental validation of the results [27]. Data simulated in the presence of an exogenous field will be compared with those obtained in the absence of the field.

We propose the adopted methodology as suitable to unveil the possible interaction mechanisms of a microwave EM field with biochemical reactions on solvated proteins.

II. MODELS AND METHODOLOGY

This section gives a brief description of the molecular model used in the simulations, the theoretical computational methods used to obtain the properties of the biochemical reaction and,

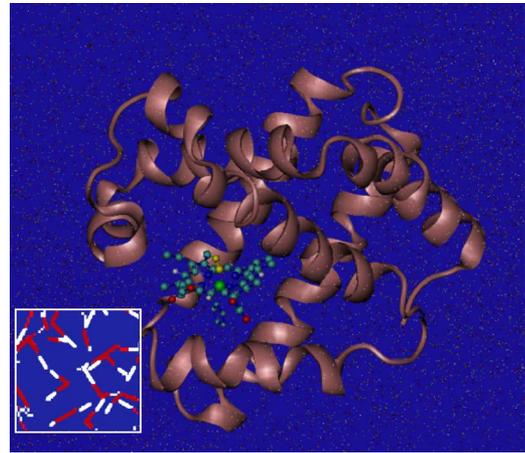


Fig. 1. Ribbon representation of the molecular model of myoglobin protein in its aqueous environment; the reaction center is represented by single atoms [iron atom (green in online version); carbon monoxide (yellow in online version)]; the 153 amino acids are represented by the seven alpha helices; in the white box, a closer view of the solvent water molecules is given [oxygen atoms (red in online version); hydrogen atoms (white in online version)].

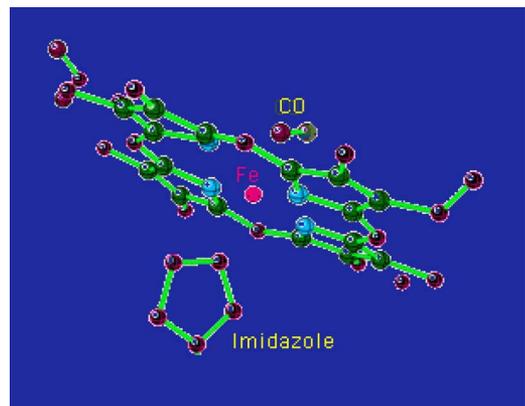


Fig. 2. Reaction center configuration (more than 100 atoms): heme group, imidazole and carbon monoxide (CO) ligand.

finally, it introduces how the microwave field was treated within this simulation context.

A. Molecular Model

Myoglobin is found mainly in heart and skeletal muscles of numerous animal species. It has a monomeric structure with only one subunit.

Sperm whale myoglobin (Fig. 1) was chosen in this study and consists of 153 amino acids that fold into a structure (seven alpha helices) that is ~ 3 nm in diameter. Although the whole protein (more than 1000 atoms) is involved in the binding process, only a piece of the protein plays the role of the binding receptor site. It is called the reaction center and determines the electronic properties of the reaction; it consists of approximately 100 atoms.

In Fig. 2, a schematic view of what we considered as the reaction center is given: the heme group (porphyrin ring) with the central iron atom, the imidazole corresponding to the proximal histidine side chain (a key myoglobin residue), and the carbon monoxide.

A number of experimental and theoretical studies have unveiled the spectroscopic properties of myoglobin in solution. In particular, absorption due to charge transfer between iron and porphyrin has been revealed in the ultraviolet and infrared range [28]–[30]. More recently, in order to clarify the relationship between the conformational fluctuations and the biological function of the protein, relevant absorption peaks in the terahertz range, due to low-frequency vibrational modes of protein subunits, have been determined [31]–[34]. Finally, theoretical [35] and experimental data [36] show conformational fluctuations of the protein occurring with time periods between a few nanoseconds down to 100 ps (quasi-diffusive motions), giving rise to significant spectral contributions between 1–10 GHz.

B. Molecular Simulations of Chemical Reactions

When dealing with molecular simulations of complex systems such as solutions and proteins, it is important to consider both the coupling between the electronic states of the reaction center and the atomic molecular environment. This last contribution is mainly linked to protein conformational fluctuations and/or water-solvent structural modifications [37], deriving from the intrinsic, not deterministic, nature of such systems.

This implies a difficult problem to solve [38] since the standard computational approaches are either classical molecular dynamics (good for the whole protein description) or pure quantum methods (suitable for the electronic properties of the reaction center). Recently, to properly study biochemical reactions, mixed and hybrid methods have been proposed in the literature. Mixed methods, like the one adopted here, imply three steps, which are: 1) classical dynamics simulation; 2) quantum mechanics calculations; and finally 3) a proper operation in order to mix the features of the previous two. In the following, some details for each methodology are given.

Molecular Dynamics: In a molecular dynamics simulation, all atoms in the system are treated classically. Each atom is considered in terms of its charge, mass, coordinates, and velocity. The simulation is based on the time-dependent numerical solution of the Newton Equation Law (1), and permits one to evaluate the atomic and molecular motions of the entire system

$$\frac{d^2 r_i(t)}{dt^2} = \frac{F_i(r, t)}{m_i}. \quad (1)$$

In (1), r_i and m_i are position and mass of each atom of the molecular system considered, while F_i is the force field, the functional form to represent the potential energy function V , used to describe the interactions between particles, and regulated by proper temperature dependencies [40]

$$F_i(r, t) = -\frac{\partial V}{\partial r_i(t)}. \quad (2)$$

The no deterministic behavior is taken into account by initial conditions on particle velocities. For each simulation, they are chosen on the basis of a temperature-dependent Maxwellian random distribution [39].

Molecular dynamics has a number of important limitations [40]. A first one is the maximum time step that allows the stability of the integration of the equations of motion. In practice,

a typical value is 2 fs, limiting, up to now, the lengths of simulations to the nanoseconds time scale, for molecular systems of 10^4 atoms. Another important limitation is the classical treatment of the system, which discards electronic properties description. This is the reason why molecular dynamics cannot be used to treat chemical reactions where the knowledge of the electronic properties of the system is fundamental.

Quantum Mechanics: Pure quantum-mechanical methods are considered as the only methods for analyzing a reaction process, given that the electronic behavior is explicitly taken into account. According to quantum theory, any physical system is described in terms of its wave function, which provides the probability to find electrons in a given space, at a given time. The evolution of the wave function in time is described by the Schrödinger equation. In many problems, the time-dependent Schrödinger equation can be simplified to its time-independent formula, which, in matrix notation, can be written as

$$\tilde{H}c_i = Uc_i \quad (3)$$

where c_i is the i th eigenvector of the Hamiltonian matrix \tilde{H} and U is the corresponding Hamiltonian eigenvalue. An analytical solution of the Schrödinger equation is possible only for the hydrogen atom, whereas in all the other cases, numerical methods are needed. Unfortunately, the accurate solution of such methods still remains accessible to very small systems because of the huge computational cost.

In the case of protein biochemical reactions, this means restricting the calculation only to the reaction center; hence, obtaining unreliable results for the chemical reaction, due to the lack of the cited contribution of the whole protein conformational fluctuations and water solvent.

Mixed Quantum-Classical Methods: Therefore, the way to accurately study chemical reactions in proteins is to use a methodology able to combine quantum and classical mechanics, in particular, here, the *perturbed matrix method* is applied [23[24]]–[26]. This methodology is based on standard perturbation theory, where the effect of a perturbation, as for example a generic electric field, can be taken into account by simply adding the perturbation term to the unperturbed Hamiltonian operator. Therefore, in the perturbed Hamiltonian \tilde{H}_p , besides kinetic and potential energy terms included in \tilde{H}^0 (the unperturbed term), there is another term related to the electric field, as follows in (4):

$$\tilde{H}_p \cong \tilde{H}^0 + \tilde{V} = \tilde{H}^0 - E \cdot \tilde{\mu} \quad (4)$$

where \tilde{V} is the perturbation term, E is the generic electric field, and $\tilde{\mu}$ is the electric moment operator.

Under the hypothesis of a perturbation sufficiently homogeneous over a rigid reaction center, the *perturbed matrix method* reduces the calculation of the electronic properties to a simple diagonalization of the Hamiltonian matrix associated to the perturbed \tilde{H}_p on a basis set represented by the wave functions of the unperturbed \tilde{H}^0 ; this operation is, of course, computationally fast.

Applying the *perturbed matrix method* to the binding/unbinding reaction in myoglobin, means calculating the electronic properties of the reaction center perturbed by the electric field

generated by myoglobin and water atomic charge distribution, with the exclusion of the reaction center atoms themselves. Such field (E_{env}) is an atomic electric field (on the order of 1 GV/m), made by two terms: the first one representing the perturbation due to each aminoacidic residue of the protein and the second which considers the perturbation due to local electric field generated by water molecules. E_{env} field is calculated by classical molecular dynamics and evaluated along the path where the ligand is moving [27], as follows:

$$E_{\text{env}}(t) = \frac{1}{4\pi\epsilon_0} \left[\sum_k \frac{Q_k}{r_k(t)^2} + \sum_j \frac{q_j}{r_j(t)^2} \right] \cos(\theta(t)) \quad (5)$$

where Q_k represents the total charge relative to the k th residue, q_j represents the total charge of the j th water molecule, r_k and r_j represent, respectively, the distance between the k th residue and the j th water molecule from the Fe atom (the center of mass of the reaction center), θ is the angle between the direction of the local atomic field (due to both residues and water molecules) and the direction along the path where the ligand is moving.

In particular, E_{env} is provided typically every $\Delta t = 2$ ps. This means that for each nanosecond of simulation, we obtain 500 samples of E_{env} ; from each of them the perturbed Hamiltonian matrix \tilde{H}_p can be constructed and diagonalized.

Finally, considering basic statistical mechanics, it is possible to derive the free energy of the chemical reaction [27], [41], [42], as the average over the samples data set of the whole simulation length, as follows:

$$\Delta A = -KT \ln \left\langle e^{-\beta(U_p - U_0)} \right\rangle \quad (6)$$

$$\beta = \frac{1}{KT} \quad (7)$$

where K is the Boltzmann constant, T is the temperature (in kelvins), and U_p and U_0 are the eigenvalues, respectively, for the perturbed and unperturbed Hamiltonian matrixes. In this way, we obtain that free energy depends on the electronic properties of the reaction center, as well as on the whole protein environment via U_p calculated through (3) with the perturbed Hamiltonian term of (4) where E is the perturbing E_{env} field of (5).

It is worthwhile to point out that the temperature dependence of the process is intrinsically taken into account by this approach.

C. Study of the Carbon-Monoxide-Myoglobin Binding Reaction With Perturbed Matrix Method

As a first step, quantum chemical calculations on the isolated reaction center (see Fig. 2) in vacuum were performed in order to obtain the electronic states. The procedure based on quantum mechanics Gamess US and Gaussian98 packages [43], [44] is described in detail in [27]. The reaction coordinate, i.e., a 1-D coordinate representing the progress along a reaction pathway, was chosen as the distance between the iron atom of the reaction center and carbon monoxide (Fe-CO). Six different distances were considered starting from a point relative to an energy minimum (located at around 2 Å from Fe) and up to 3.8 Å. At each

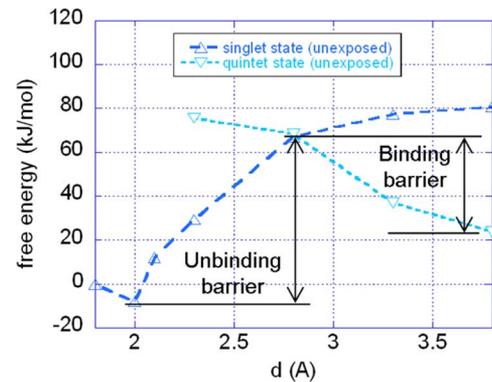


Fig. 3. Myoglobin free energy as a function of the reaction coordinate ($d = \text{Fe-CO}$ distance) in the unexposed situation for the singlet and quintet states of the molecule.

distance the unperturbed Hamiltonian can be calculated and the energy of the system provided. This procedure was performed for the two magnetic states of myoglobin: singlet and quintet magnetic states.

Secondly, molecular dynamics simulations were performed at 300 K, constraining myoglobin-CO in the center of the simulation box (around 6-nm side), filled with 6741 water molecules (Fig. 1), at a liquid density (49.1 mol/L) determined by an initial 1.0-bar isobaric-isothermal equilibration run. Note that, to properly describe myoglobin physiological behavior, it was necessary to simulate a box of water molecules large enough to reproduce both the first hydration shells [17] and the remaining bulk water (Fig. 1, closer view in white box). All the simulations were performed using the Gromacs package [39].

Finally applying the entire procedure explained in Section II-B, we derived the free energy at each distance Fe-CO and for both magnetic states, giving rise to the curves reported in Fig. 3.

The barrier for the binding process, which is the energy that must be overcome in order for the binding chemical reaction to occur, is given by the free-energy difference between the value at 3.8 Å, where CO is unbound, and the value where the singlet and quintet curves intersect.

Likewise, the barrier for the unbinding process is provided by the difference in free energy between the crossing point and the one where CO is bound ($d = 2$ Å, free-energy minimum). Therefore, for each simulation, we obtain a binding and unbinding barrier value. Such barriers may be denoted as the minimum energy to make possible a specific chemical reaction to occur. Binding and unbinding barriers are related to chemical kinetics and, in particular, to the reaction rates of a chemical reaction, from which rate laws and rate constants can be derived.

It is worth noting that the use of explicit water molecules within the simulations guarantees that all the possible interactions (i.e., protein-water, ligand-water, ligand-protein) are considered in the calculations and that all the electrostatic effects are taken into account. Therefore, no macroscopic description of the dielectric medium in terms of permittivity value has to be considered.

D. Introducing Microwave EM Fields

In this paragraph, the problem of introducing an exogenous EM field in the molecular simulations is faced. On this purpose, some considerations have to be drawn.

The first one regards the frequency of microwave radiation. As known, energy of microwave frequency quantum falls very far below excitation and ionization energies of the reaction center (energy ratio $\sim 10^{-5}$); therefore, microwave fields may not induce ionization. Looking at the intensity of the field, it is possible to state that to alter the electronic structure of the reaction center, a very intense electric field (100 MV/m) have to be applied, as clearly shown in [22]; therefore, a direct action of microwave fields of lower intensities on the isolated reaction center, in vacuum, is not feasible.

In spite of this, a possible microwave effect could be exerted by an indirect mechanism involving modifications of protein atomic motions, having a spectral contribution between 1–10 GHz, as explained in Section II-A. Therefore, the possible coupling of the microwave field with protein internal motions and water molecules and its influence on the reaction can be investigated.

For these reasons, the microwave signal was introduced in the molecular dynamics simulations. In this way, we are able to take into account not only microwave–protein/ligand interactions, but also microwave–solvent (water). Such analysis seems to be feasible since typical duration lengths of such simulations (tens of nanoseconds) are consistent with some periods of the applied microwave signal.

As the Gromacs package, in its standard version, does not support the presence of continuous wave electric fields, the code was modified by introducing a suitable routine, which allows adding an oscillating field to the standard force field provided by the package. The microwave field was represented by a homogeneous time-alternating electric field of the form

$$\mathbf{E}_{\text{ext}}(t) = E_0 \cos(\omega t) \mathbf{x}_0 \quad B = 0 \quad (8)$$

as suggested also in [45] and [46]. Therefore, the basic equation (2) of molecular dynamics becomes

$$m_i \frac{d^2 r_i(t)}{dt^2} = F_i(r, t) + q_i \mathbf{E}_{\text{ext}}(t). \quad (9)$$

In this way, the external microwave field, acting as an additive force on each atom of protein and water solvent, modifies single atom positions; hence providing, by (5), the E_{env} perturbing the reaction center recalculated in presence of the microwave electric field. After this, by means of the procedure outlined in Section II-B, it is possible to extract the binding/unbinding barriers involved in the reaction in the presence of the external field.

In order to validate the insertion of the microwave field in the molecular dynamics simulations, the action of a 1-kV/m 1-GHz microwave field has been considered on an ion of charge q and mass, in vacuum. This means that in (9), $F_i(r, t)$ was considered null. Fig. 4 reports such results compared to analytical calculations, showing perfect agreement, indicating that the external field was acting properly in our simulations.

In the following, results obtained under the presence of a microwave field will be referred to as exposed conditions data,

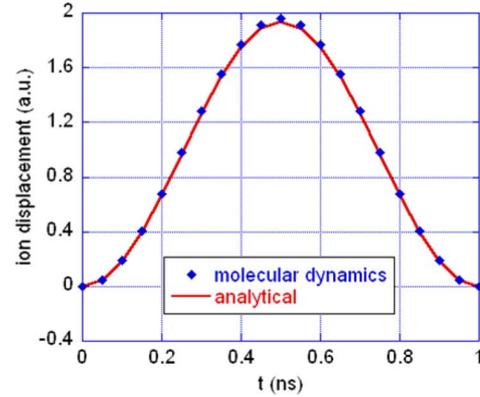


Fig. 4. Single ion (Fe^{2+}) in vacuum subject to an applied electric field (1 kV/m at 1 GHz): comparison of molecular dynamics simulation and the analytical solution of Newton’s Law.

while unexposed condition refers to the simulated molecular system with E_{ext} set to zero, but still in the presence of all the other forces.

III. RESULTS

An unexposed simulation of approximately 10 ns, the same time length used in previous results [27], gave the data reported in Fig. 3. Such a time duration is considered sufficient for a complete statistical exploration of the configurational states of proteins [38]. Values for binding and unbinding barriers (mean \pm standard deviation) were 41.9 ± 0.8 and 73.7 ± 0.5 , respectively. These data are in good agreement with those reported in [27]: 43.8 ± 0.6 (binding barrier) and 74.1 ± 0.9 (unbinding barrier), where an acceptable comparison with experimental data was performed [27, p. 644].

A similar graph has been obtained for an exposure condition of 1 GHz with a field amplitude of 1 kV/m from a simulation performed for 40 ns after an initial equilibration. Such a longer time duration was chosen since the behavior of myoglobin under exposure conditions was not *a priori* known. This case provided a value for the binding barrier of 44.5 ± 0.6 and for the unbinding one of 71.5 ± 0.9 .

Due to the stochastic properties of such systems, taken into account as previously explained, we created a statistical ensemble, in order to provide a reliable comparison between the system not exposed to the microwave field and the system exposed to the fields. The use of data obtained by a few simulations of insufficient time duration may result in apparent enhanced variations due to statistical noise [39].

Moving from the idea that molecular simulations can be considered as “virtual experiments,” we used statistical analysis in order to compare the mean values of the two populations (not exposed and exposed) in seeking some differences. A statistically significant difference in the barriers of the exposed and unexposed simulations would imply an unbalancing of the reaction in one of the two directions: helping or opposing to the binding of carbon monoxide to the myoglobin. In this way, we are able to investigate if microwaves can alter the process of carbon monoxide removing from the muscle tissues by the myoglobin.

TABLE I
FREE ENERGY BARRIERS IN UNEXPOSED AND EXPOSED
CONDITIONS (1 GHz, 1 AND 10 kV/m)

	Binding barriers (kJ/mol)	Unbinding barriers (kJ/mol)
Unexposed		
unexp. #1	43.5	74.0
unexp. #2	43.1	74.5
unexp. #3	42.4	73.8
unexp. #4	41.9	73.7
unexp. #5	43.9	73.0
mean±S.E.	42.9±0.4	73.8±0.2
Exposed 1 kV/m		
exp. #1	45.4	72.7
exp. #2	42.2	74.4
exp. #3	43.0	74.1
exp. #4	40.4	74.5
exp. #5	43.8	73.5
exp. #6	43.6	73.5
exp. #7	44.4	73.0
mean±S.E.	43.2±0.6	73.6±0.3
Exposed 10 kV/m		
exp. #1	41.8	74.5
exp. #2	43.6	73.5
exp. #3	43.2	73.5
mean±S.E.	42.8±0.5	73.8±0.3

For these reasons, we have evaluated the reaction properties via a set of independent simulations: five under unexposed conditions, and ten under exposed conditions, seven for amplitudes of 1 kV/m, and three for 10 kV/m. The simulations were performed for at least 10-ns time periods, whereas some were prolonged up to 20 ns, implying a computational effort of approximately 36 h for a single simulation of 10 ns, running on a cluster of 20 Xeon at 1 GHz, equipped with 1-GB RAM memory each.

Results for the energy barriers in the exposed and unexposed cases are given in Table I. Regarding the unexposed data, we can observe for the binding barrier (mean value ± standard error) 42.9 ± 0.4 kJ/mol and for the unbinding one 73.8 ± 0.2 kJ/mol, averaged over the five values obtained from the unexposed simulations. The low intrinsic variability in the unexposed barriers (~ 2 kJ/mol), corresponding to less than 5% of the binding barrier value and less than 3% of the unbinding one, confirms that myoglobin is an extremely stable protein with low sensitivity to its temperature-dependent random initial conditions.

Concerning the exposed results, it can be observed that the binding and unbinding barriers in the presence of microwave electric field intensities of 1 and 10 kV/m have a greater variability with respect to the unexposed ones. Nevertheless the statistical analysis (unpaired student t-test), based on the assumption of a Gaussian distributed noise, confirms the absence of any statistically significant effect.

Further simulations with increasing electric field amplitudes were performed, up to a maximum field intensity of 100 MV/m, and no significant variations in the reaction free-energy profile have been observed. Therefore, even for the whole protein, as with the isolated reaction center, 1-GHz microwave fields with intensities below 100 MV/m seem unable to affect the biochemical reaction studied.

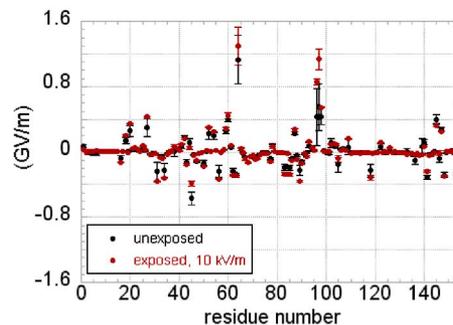


Fig. 5. Mean perturbing electric field (E_{env}) projected onto the reaction coordinate (Fe–CO distance) for each of the residues of the protein included in the solvent for the unexposed and exposed 10 kV/m.

Once excluded a relevant effect of the microwave EM field on the reaction properties, it was still interesting to investigate the possible effects induced by the exogenous field on single protein residues and water solvent. In Fig. 5, we show the values of E_{env} , due to each protein residue and water molecule, averaged over the number of simulations, both for the exposed 10-kV/m (three simulations) and unexposed cases (five simulations). The figure indicates that only a few slight deviations are present, but not statistically significant (unpaired student t-test).

Conversely, for high-intensity electric fields, changes in the protein conformations were observed. The parameter used was the *root mean square deviation* (rmsd) of the solvated protein obtained by least square fitting of the structure with respect to a reference one: it can be calculated as in [39]

$$\text{rmsd dev}(t_1, t_2) = \left[\frac{1}{M} \sum_i m_i \|r_i(t_1) - r_i(t_2)\|^2 \right]^{1/2} \quad (10)$$

where

$$M = \sum_{i=1}^N m_i. \quad (11)$$

m_i represents the mass of each atom and $r_i(t)$ represents the position of atom i at time t .

For a single simulation of 500 MV/m, despite no significant variations in energy barriers, a value of *root mean square deviation* three times higher than the unexposed one is obtained, congruent with data reported in [46]. This is considered an initial localized denaturation of the protein due to modifications in protein alpha helices [46].

IV. DISCUSSION AND CONCLUSIONS

In order to clarify the possible effects of microwave EM fields on biological systems, we investigated the effects of exposure on the carbon-monoxide binding/unbinding process to myoglobin, considered as a prototype reaction for many important biochemical processes.

The investigation of possible molecular mechanisms of microwave effects on protein behavior has been largely examined in the last years. There is substantial evidence that effects on bio-molecules in solution and living tissues due to high level

of microwave radiation (some kilowatts/kilogram for the specific absorption rate) cannot be entirely reduced to macroscopic heating [47].

A recent review [48] collects many experimental *in vitro* studies regarding microwave nonthermal induction of heat shock proteins concluding that the possibility that microwave exposure might have a direct effect on proteins should be considered a working hypothesis. Moreover, the existence of a microwave effect on folding/unfolding of the β -lactoglobulin has been argued from experimental results by Bohr and Bohr [49]. In George *et al.* [50], it is shown that microwave exposure of a protein (Citrate Synthase) causes a significantly higher degree of unfolding than conventional thermal stress for the protein solution heated to the same maximum temperature. Therefore, the possibility that such types of effects are due to a direct action of high-exposure intensities on protein conformation, resulting in significant changes in the biological function, is plausible. Our results in changes of *root mean square deviation* for very high intensities are in accordance with such observations.

Besides an action of microwaves on protein conformation or solvent, the existence of specific (nonthermal) microwave effects on chemical reactions has been often postulated [14], [19], [20] even if microwave activation has been demonstrated only for solvent-free compounds [51].

However, on the basis of numerous experimental studies, it is concluded that more computational calculations and development of theories are still required [20], especially when dealing with reactions typical of protein complexes.

In this study, molecular dynamics simulations combined with quantum calculations and statistical mechanics were applied to describe the binding–unbinding reaction of carbon monoxide to myoglobin in the presence of a microwave field. The results obtained were compared to the reaction behavior, studied in the same way, in the unexposed molecular system.

In order to reduce the intrinsic statistical noise related to the calculation of the essential properties describing the chemical reaction (the free energy barriers), we compared many simulations in both unexposed and exposed conditions. Results clearly show that microwave fields (1 GHz and electric field intensities below 100 MV/m) are unable to alter the structural fluctuations of the protein, and hence, the reactivity of the binding/unbinding process.

Such results, although indicating that in similar and/or related biochemical reactions microwave fields are likely to be irrelevant, cannot be straightforward extended to different biochemical reactions in proteins due to the peculiar structural properties of globin family, which provide a high functional stability to myoglobin [18].

Nevertheless, the methodology followed is robust to investigate protein behavior and biochemical reactions, and shows a possible way to study the basic mechanism of microwave bio-effects, investigating at the basis of the possible molecular transduction of the field. In the meantime, it guarantees a rigorous approach extensible to other microwave research fields like industrial applications of microwave chemical reactions and to the new emerging area of nanotechnological applications.

ACKNOWLEDGMENT

The authors want to thank A. Chiabrera, Italian Inter-University Centre for Electromagnetic Fields and Biosystems (ICEmB), Genova, Italy, for his strong encouraging on molecular dynamics research, G. Vecchi, Politecnico Torino, Turin, Italy, who provided useful indications for the final version of this manuscript, and M. H. Repacholi, University of Rome “La Sapienza,” Rome, Italy, for his precious suggestions in manuscript finalization.

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