Current Topics in Peptide & Protein Research manuscript No.
(will be inserted by the editor)

Fabrizio Mancinelli · Emiddio Di Maggio · Ettore Bismuto

Myoglobin as model protein to investigate non-thermal effects of mobile phones electromagnetic fields

the date of receipt and acceptance should be inserted later

Abstract For almost two decades scientific community has questioned about possible health risks due to microwave electromagnetic fields (MWEMF) exposure caused by mobile phones usage and the related environmental "electromagnetic pollution". Investigations have been carried out in many directions, especially concerning about in vivo experiments involving cells, tissues or animal models. Other researchers have focused their attention on relatively "simpler" models, such as a protein, considered as the basic brick of all physiological processes, no matter how complicated they are. Authors have differentiated either in the choice of exposure systems, which range from waveguides to resonating point-like applicators, and in the protein chosen as model, with a common preference for small and well described molecules. Some reports have evidenced significant non-thermal effects on proteins behaviour, which can be with more than a reasonable likelihood ascribed to MWEMF perturbation. Our group is working for years about effects of MWEMF on tuna apomyoglobin. This well-known protein present a bulk of spectroscopic observables, which make it very suitable for studying its conformational dynamics in particular environmental conditions. At first we have checked whether exposure to low powered MWEMF causes loss of secondary structure and function capability of apomyoglobin in solution, observed by molecular absorption and circular dichroism, as well as changes in its conformational dynamics analyzed by measurements of fluorescence lifetime of apomyoglobin sole tryptophan residue; no remarkable effects have been found in this situation. Afterwards, we have formulated the hypothesis that, rather than a formed and native structure, a better candidate to undergo changes in some of its properties due to an external electromagnetic perturbation is a partially refolded structure, whose more loose motions may have a greater likelihood of resonance at these frequencies. Therefore we have studied possible non-thermal MWEMF effects on apomyoglobin in its acidic state: in this case, an alteration in the refolding process has been observed, together with a different population of the conformational substates among which the protein fluctuate. The alteration in the refolding process could have repercussions on the competent aggregation process, with formation of amorphous oligomers which could resonate even more at MWEMF frequencies. This conjecture seems to be addressed by computational studies we have carried out about apomyoglobin conformational dynamics, which show how the widest backbone motions frequencies of small oligomers fall in the range of microwaves. All these results would open a very interesting landscape, since many modern social diseases are caused by misfolding/aggregation processes.

Keywords Microwaves non-thermal effects · myoglobin · frequency domain fluorometry · molecular dynamics

F. Mancinelli, E. Di Maggio, E. Bismuto
Dipartimento di Biochimica e Biofisica, Seconda Università degli Studi di Napoli, Naples, Italy
Tel.: +39-081-5667635
Fax: +39-081-5665863
E-mail: ettore.bismuto@unina2.it

1 Introduction

The last decade has seen a so huge improvement in the radiocommunications, that this could be called without any doubt a revolution. Mobile phone users have been calculated [1] to be about a fifth of the whole world population, a number probably underestimated and destined to grow even more. The development of such techniques adds to the communication devices which already over decades crowd the aether, inducing to coin the term "electromagnetic pollution".
As a consequence, there has been an increasing concern by the public opinion about possible health hazard due to electromagnetic fields exposure [2]. In particular, wide and often controversial discussion in the scientific community have arosen about potential damages induced by low-powered microwaves (MW) exposure; such effects are called "non-thermal" or "athermal", in order to distinguish them from the heating effects observed at higher power.
A huge bulk of experimental work has been performed, in order to search for correlations between exposition to MW fields generated by handphones and biological effects, of functional and pathological interest, on humans and other living organisms. Aside epidemiological studies [3,4], researchers have evidenced, among others, non-thermal MW effects concerning alterations of cell response to oxidative stress [5], modifications of catalytic efficiency of some enzymes [6,7], variations of ionic transport flux through specific membrane channels [8], DNA damage [9], embryonal growth irregularities [10], and brain cancer promotion [11]. Deep interest has been also arosen by some observations demonstrating an "heat shock response" following prolonged exposure to low-powered MW irradiation, that appears to be non-thermal [12–14]. The heat shock response is mediated by the "heat shock proteins" (HSP), a class of macromolecules which probably act as "molecular chaperones" involved into recovery of damaged proteins.
The complexity of the investigated systems and the variability of the experimental conditions, especially related to the perturbing field features (exposure duration, operating frequency, power, signal modulation), make arduous to perform experiments in a reproducible manner. This could explain contrasting findings about non-thermal effects of microwaves on similar if not equal biological samples. For example, an experiment [15] carried out on Fisher 344 rats exposed to a frequency-modulated 836.55 MHz field, simulating head exposure of a mobile user, revealed no effect on spontaneous and nitrosurea-induced tumors of nervous system; this negative findings contrast with a previous, analogous study [16]. Other examples of controversial results concern an increased susceptibility of transgenic mice (Eμ*Pim1*) to develop lymphomas as consequence of long-term exposure at 900 MHz radiation [17,18], or spatial-working memory deficit in rats exposed to low-level MW radiation [19,20]. In the latter couple of experiments, while the first paper hypothesizes an effects of MW on both cholinergic and opioid systems within the brain, the second concludes that there is no evidence about possible influence of microwaves exposure in the ability of rats to complete a maze task.
Aside the experimental work, there are also some reports which, starting from theoretical considerations, explore the possibility that biological systems coupled to electromagnetic fields may exhibit classical resonance at MW frequency, thus affecting functional properties [21,22]. Even in this cases, the conclusions are controversal; but has to be remarked that biomolecules are complex structures which possess a large variety of intra- and inter-molecular interactions. Moreover, as concerns proteins in particular, they are provided with a hierarchical organization of internal motions whose extent ranges from few atoms up to whole segments of the structure.
Due to this considerations, many researchers have recognized in protein macromolecules proper biological models to investigate this debated matter. A former, pioneering work in this subject was been carried out by Bohr and Bohr in 2000 [23]. They have suggested that microwaves may cause conformational effects through a stimulation of the coherent intrinsic dynamics in biomolecular chain molecules like proteins. Such behaviour is consistent with the view that protein folding is the result of a competition between torsional and bending forces, and not only driven by entropy [24,25]. Folded structures that are stabilized by hydrogen bonds, disulfide bridges, etc., can mantain significant fraction of folding in balance with unfolded chains. Because of topological constraints, folded and unfolded proteins can possess an intrinsic dynamics with collective nature, whose lower eigenmodes are in the microwave range of approximately few GHz, and the molecules may therefore be excitable by microwave irradiation.
Bohr and Bohr's experiment present a convincing evidence that microwave irradiation can affect the kinetics of the folding process of the globular protein β-lactoglobulin dissolved in urea solution and

acidic pH: in this solution the protein can be, depending on temperature, either in cold denaturation state, or in folded state, or in hot denaturation state.
At low temeprature the folding from the cold denatured phase of the protein is enhanced, while at a higher temperature the denaturation of the protein from its folded state is enhanced. In the latter case, a negative temperature gradient is needed for the denaturation process, suggesting that the effects of the microwave are nonthermal. The refolding of the protein has been monitored by the optical rotation dispersion (ORD) technique, which measures the polarization. The microwave radiation for this experiment was provided from a standard magnetron of a microwave oven operating at 2.45 GHz and with a power of 800 W; power absorbed per mass unit by the sample (Specific Absorption Ratio, SAR) has been calculated as about 250 mW/g. Due to high resonance of microwaves with water molecules roto-vibrational motions, it may occur even intense heating. However, thermal baths ensure temperature control in order to properly uncouple heating effects from alleged non-thermal effects.
In this connection, it has to be noticed that an ordinary microwave oven, though allows the control, by means of proper devices, of the power absorbed by the protein solution, could not give any information about the field distribution into the sample, and therefore could not guarantee its uniformity. A way to overcome this problem may be to irradiate a sample volume which is small enough to consider the field inside almost constant.
In a recent paper from Copty et al. [26], authors have made use of a near-field applicator to irradiate the sample, a solution of enhanced green fluorescent protein (EGFP) of which they have monitored the fluorescence under microwave exposure. With their device, authors obtained a highly focused microwave spot whose size is less than 1 mm, which is the order of magnitude of microwaves wavelenght. However, the applicator's operating frequency is at 8.5 GHz, which is rather far from the frequency utilized for mobile communications. Nevertheless, their results are very interesting in showing non-thermal effects of microwave exposure on fluorescence. Authors compare reduction and red-shift of fluorescence they observe to the same effect generated by simple temperature rise; they conclude that the reduction is not only power-dependent, but it could not be assigned to bare heating. However, they do not propose any hypothesis about a "window effect" of microwaves as resonances with collective motions of the protein itself. Rather, they put their attention on the layer of bound water around the protein molecule, which has an absorption peak right around the operating frequency in their experiments, i.e. 8–9 GHz [27], while bulk water absorbs at higher frequencies. They therefore interpret their results as due, at least partially, to a kind of "local heating", which may affect protein folding.
A recent paper from Weissenborn et al. [28] casts more doubts on the likelihood that microwaves may couple non-thermally to globular proteins at hydration levels at which they still function. They have carried out measurements of X-ray diffraction (XRD) of tetragonal single crystals of hen egg-white lysozime exposed to microwave fields at 8 GHz delivered by a modified slab-line waveguide. At high microwave power level (about 3 W), increased but recoverable lattice defects have been found, mainly owing to the evaporation of crystal water. At lower microwave power levels (about 1 W), the perturbing external field produced changes in the mean-square displacement (B factors), which they compare to similar results due to bare heating. From this comparison, they conclude that microwave are resonantly absorbed not by the protein macromolecule itself, but rather by unbound crystal water. Moreover, they hypothesize that owing to the presence of water in globular proteins, intramolecular vibrations at microwave frequencies should be strongly damped.
From this large amount of data, appears therefore evident how necessary is to frame all the observations in a rational scheme, in order to trace a way for carrying out further investigations which are comparable each other, to finally establish whether long-term mobile phone exposure is health hazardous.
In this paper we present our long-termed and wide-featured research on *Thunnus thynnus* myoglobin chosen as model protein to investigate this debated matter. The experiments provide a leading evidence of interactions between macromolecules and perturbing external fields, and suggest a number of observables to consider in planning an experiment. To provide a theoretical background in order to clarify physical mechanisms of such interaction, we have taken into account fast-growing computational methods which give an unsubstitutable deeper view into the molecular and atomic details of the protein dynamics, looking for possible resonances between their internal-reciprocal motions and a perturbing oscillating external field.

2 Myoglobin: a suitable model

In the universe of proteins studied for their structural and functional properties, myoglobin takes its place at the highest seat. There are in fact many reasons to consider this small all-α globular protein one of the best candidates to handle for investigations about structure-function relationship, in physiological or altered conditions:

1. Myoglobin was the first protein whose 3-D structure has been resolved by means of crystallographic X-ray diffraction.
2. Myoglobin was the first protein in which there has been recognized a complex hierarchical level of internal motions, since the very first observations that the crystal structure did not highlight an input channel into the heminic site for the oxygen molecule. Thermally induced conformational fluctuations allow the binding to molecules of oxygen and other ligands or their release [29,30].
3. The presence of several spectroscopic observables in the same protein, i.e. the absorption bands of heme group in the visible spectral region, the excitonic absorption in the far-UV due to the polypeptide chain, and the near-UV absorption of aromatic residues.

All these features have made myoglobin widely studied by spectroscopical techniques; in fact, these kind of observations are very effective in giving information on its structural organization and its conformational dynamics [31–33]. Moreover, the spectroscopic investigations in the absorption region of heme group will show possible effects of the MW field on the heme-apoprotein interaction; in particular, it is possible to evaluate its functional capabilities by monitoring the bond with ligand molecules at high affinity as CO [34].
Among the several myoglobins present in the animal kingdom, the one from *Thunnus thynnus* has a special feature which makes it peculiarly suitable for advanced spectroscopic studies: it possesses in its aminoacidic sequence a sole tryptophan residue. The conformational dynamics of a given protein are effectively pointed out by the analysis of the intrinsic fluorescence emissive decay of single tryptophan proteins [35,36]. Usually, tryptophan fluorescence lifetimes are observed, rather than a single value, as a quasi-continuous distribution, whose mean value is related to the microenvironment experienced on average by the fluorophor in the excited state inside the protein matrix. The width of the fluorescence lifetimes distribution is a measure of the heterogeneity of the several environments existing in the variety of conformational substates accessible to the protein at the given temperature.
An active myoglobin molecule may fluctuate among a large number subconformations almost isoenergetic. Conformational transitions between different forms represent the fundamental link between structure and function [29]. Myoglobin fluctuations occur around the native architecture which is determined by stabilizing interactions, among which electrostatic forces play an important role [37]. This suggests that the action of an oscillating electromagnetic field may induce forced vibrations in the protein structure able to influence its conformation. In particular, it has also been calculated that the region surrounding the heme binding site has a negative charge density which may be perturbed by an exciting MW field; this perturbation could modify the accessibility of this functional region to water or other lingand molecules.

3 What are the observables?

As already highlighted, *Thunnus thynnus* myoglobin has many spectroscopic observables in its physiological state; so, first experiments were performed on protein solution in native conditions [38]. The optical density spectrum of myoglobin reveals in the visible region a strong absorption band peaked at 406 nm, known as *Soret band*, when the iron atom is at the oxydation state III and the sixth ligand position is a water molecule (met-myoglobin). The position and width of this band are very sensitive to changes in the heme interaction with the surrounding protein. The exposure system was a waveguide-featured, in which an unmodulated, 1.95 GHz frequency TE field delivered a SAR of 51 ± 1 mW/g for a duration of 2.5 hours on a cuvette containing 3 mL of myoglobin solution. In order to ensure that the power was delivered uniformly throughout the most of the exposed volume, calculations of the field inside the volume were carried out by means of specific software CST Microwave Studio which numerically resolves Maxwell's equations in the given space. The "inhomogeneity index" (SAR standard deviation/mean SAR) was found to be 0.49.
The presence of MW field did not cause any alteration in the aspect throughout the whole Soret

spectral region; this result was also found to be concentration-independent (figure [38]). The Soret region is a good observable also for monitoring the myoglobin main function, that is carrying oxygen molecules. However, the affinity of myoglobin in the ferrous state for carbon monoxide is about 200 times greater than for oxygen, which make CO more feasible to test myoglobin binding capability; as a consequence, the absorption maximum red-shifts to 423 nm [34]. However, even microwave exposure on CO-myoglobin samples did not cause significant spectral changes, suggesting that MW radiation does not cause any effect on the ligand binding function of myoglobin.
The measurements of CD spectra as a tool to investigate the structural organization of protein molecules is of particular advantage for myoglobin. These molecules contain, in addition to the protein moiety, the heme, whose electronic transitions are quite intense and very sensitive both to the surrounding environment and to the ligand binding. This situation offers three distinct regions of investigations, each containing information concerning a part of the structural organization of the myoglobin molecule, as previously highlighted. In detail: the far-UV region (190–240 nm) is sensible to changes into the secondary structure [39]; the near-UV (240–300 nm) is dominated by the optical transition of the aromatic residues, whose reciprocal positions gives information on local chain-chain interactions [40]; the visible region above 300 nm, in which CD spectra of the heme transitions are governed by the asymmetry of the protein environment. Therefore, the optical activity of myoglobin spectra depends on the relative positions of the different chromophores in the three-dimensional organization of the molecule [41]. Alteration of the relative positions in different conformational states are likely to cause alterations in the interactions from which the CD spectra of the different chromophores derive. This is the reason why the CD bands could be correlated to specific structural aspects and changes induced by the presence of the MW field.
Exposure experiments carried out on myoglobin [38] indicate that neither the secondary (essentially α-helical) structure, nor the tertiary structure at least for the aromatic residues surroundings, are affected by the MW field at working frequency. Moreover, the absence of alterations in the Soret absorption band seems to exclude any resonance coupling between the perturbing MW field and the contacts of heme with the rest of the protein matrix.
While all these spectroscopic observables are about "instant pictures" of the relative features, a more dynamic view could be supplied by the analyis of the tryptophanilic fluorescence decay lifetime, which as already noticed is a measure of the conformational dynamics of the fluorophor neighbourhood. The data collected, both for exposed and not-exposed apomyoglobin samples, may be treated as multi-exponential as well as distributional decay [42,43]. In all cases, the most appropriate model to best fit the emission data appears to be a unimodal lifetime distribution on the basis of the χ^2 values. In this picture, the mean value is related to the microenvironment experienced on average by the fluorophor in the excited state inside the protein matrix, while the width of the fluorescence lifetime distribution is a measure of the heterogeneity of the several environments existing in the variety of conformational substates accessible to the protein at the given temperature. Table 1 reports the lifetime analysis; as it is evident, whatever is the model chosen, parameters for both irradiated and non-irradiated apomyoglobin molecules appear to be rather similar, suggesting that in native conditions the spectrum of the conformational substates accessible to apomyoglobin molecules are not modified by the MW exposure.

4 Adding flexibility: the acidic state

Protein macromolecules undergo a maturation process during their transduction from mRNA's, to which usually one refers as *folding*. Folding of globular proteins is a very complex process involving collapsed conformations, such as the "molten globule" state, a supposed very general intermediate occurring in the protein folding pathway [44,45]. It has been found [46–48] that at acidic pH and in presence of salt myoglobin molecules fluctuate fast among unfolded and partly folded states with characteristic of molten globule, i.e. a native-like architecture but with labile tertiary interactions. This unfolding process is, at least partially, reversible: bringing the conditions back to physiological pH, these acidic forms of myoglobin with α-helix content migrate to the folded state, the partly formed secondary structure elements pack against each other with large hydrophobic contact areas so reconstituting the heme binding site. This is quite similar to what comes out during the initial stages of myoglobin folding.
Actually, the refolding process is not completely reversible, because it competes with the aggregation

process to which each protein, under suitable external condition (concentration, pH, presence of salt, temperature), is subjected. Moreover, the protein regions that are susceptible to fluctuations can be even more susceptible to aggregation processes by nucleation-growth mechanism [49,50]. Several neurodegenerative diseases have been associated with the unfolding process and subsequent fibrillization of proteins, among which the so called "amyloid pathologies" such as prion disease [51].
In acidic pH conditions, myoglobin molecules possess a net positive charge distributed along the polypeptide chain [54]; moreover it is an intrinsically flexible structure [29,37] performing local motions but also involving large scale structural rearrangements. Since protein machines are fluctuating structures having their own electric charges, their motions involved in many basic functions like catalysis, regulation, transportation, and aggregation [52] could be triggered by their interactions with MW-EMF. Specifically, the vibrational modes in the protein macromolecule could couple to an external oscillating electromagnetic field, although random thermal agitation is overlapped to the excited resonances in protein macromolecules causing a possible masking of the effects by exposure to low-level RF fields [21,22].
These considerations have suggested the idea of investigating the effect of microwaves at 1.95 GHz on the refolding kinetics of the heme binding site starting from acidic compact state of apomyoglobin (i.e., 50 mM sodium phosphate, pH 3.0). In such conditions, the classic Soret absorption spectrum, that has a maximum at 406 nm, blue-shifts with a maximum at 385 nm, because of the heme binding site disruption and the release of the prosthetic group in the solvent. Utilizing the same exposure system of native state experiment, authors have subjected to a 2.5 hours microwave exposure an acidic myoglobin solution sample. After exposure, samples were abruptly brought back to neutral conditions by fast mixing of additional solvent. Refolding was followed monitoring optical density at 406 nm; the experiment was carried out in different time windows after exposure. As can be seen in figure 1, microwave exposure slowers the refolding capability of myoglobin molecules.
It has already noticed that myoglobin refolding is a multistep process which competes with irreversible events related to protein misfolding and aggregation [55]. The irreversibility in the refolding process is time dependent as shown in the same figure 1, where the increasing absorption in the Soret region indicates the reconstitution of heme pocket of myoglobin native structure; as shown in this figure, only a small amount of acidic protein is able to form intact heme binding site after 8 h at pH 3.0 and 30 °C. In order to give a quantitative evaluation of the effect, data were best-fitted to a bi-exponential model, whose resulting parameters are summarized in table 2. Even if it is difficult to attribute a molecular significance to these parameters, statistical significance tests [56] carried out on the data allow to give a reasonable evidence that the reconstitution of the heme pocket could be altered by the presence of an external MW field.
As an additional and important measurement to better characterize the acidic state, an analysis of the tryptophanil fluorescence decay of apomyoglobin has also been performed, both for exposed and non-exposed protein solutions. The data collected from several independent measurements, were globally analyzed by nonlinear least squares routines using algorithms for multi-exponential as well as distributional analysis by linking the fit parameters [42,43]. In all cases, the most appropriate model to best fit the emission data appeared to be a uni-modal lifetime distribution on the basis of the χ^2 values. According to this model, the mean value of the lifetime distribution is related to the microenvironment experienced on average by the fluorophore in the excited state inside the protein matrix, while the width of fluorescence lifetime distribution (FLD) is a measure of the heterogeneity of the several environments existing in the variety of conformational substates accessible to the protein at the given temperature. In the figure 2 are represented the tryptophanil fluorescence lifetimes for both exposed and non-exposed samples. The distributions differ from each other slightly in their mean value, but more evidently in their width. In particular, exposed samples FLD is narrower compared to non-exposed.
To interpret these results, it is useful to refer to the studies carried out on the partially folded states of apomyoglobin by NMR spectroscopy and stopped flow CD [57,58]. Englander [59], in examining protein folding intermediates by hydrogen exchange kinetics, identified three kinds of barrier processes: (a) an initial intrinsic search-nucleation collapse process that prepares the chain for intermediate formation by pinning it into a condensed coarsely native-like topology; (b) smaller search-dependent barriers that put the secondary structural units into place; and (c) error-dependent misfold-reorganization barriers that can cause slow folding, intermediate accumulation, and folding heterogeneity. Since under weakly folding conditions the polypeptide fluctuates between these unfolded states, the presence of an external

MW field could decrease some energy barriers, altering the conformational substates distribution. Once the perturbation has removed, molecules are "entrapped" preferably in some conformational substates more than others, from which could be more difficult (that is, "unlikely") to escape to reach the folded state; this could explain both refolding kinetics and fluorescence lifetime analysis data.

5 A deeper insight through MD simulations

In the computer era, technological improvements and enormously growing computational power have provided to researchers, involved in biomolecular studies, an unsubstitutable tool for analyze even at atomic detail level protein dynamics and other biochemical processes. What a decade ago was looking impossible, like following protein motion up to the microsecond scale, is now quite affordable with molecular dynamics (MD) simulations techniques. Algorithms used in such techniques allow, under certain approximations, to numerically resolve Newton's equations relative to the atoms constituting a molecule, starting from its crystallographic structure, in order to follow e.g. the protein dynamics into a "trajectory".
To obtain from the trajectory informations about protein function is often necessary to identify collective and coordinated motions between specific structure districts. This operation is not always easy, because such motions could be "masked" by high frequency fluctuations of single atoms. *Normal Modes Analysis* is a powerful technique, based on MD simulations, which is very useful in studying "slow" correlated motion in large biomolecular structures such proteins [60,61]. Normal modes are essentially coupled vibrations between atoms of a protein whose potential energy is in a local minimum: in such conditions it is reasonable to adopt the *harmonic approximation* which allows to treat in an analytical manner (that means *exact*) the calculations. Without entering in tedious computational details which can be found elsewhere [62], performing a normal modes analysis on a protein structure allows to extract from the motion of the molecule around a local energy minimum, $3N-6$ collective coordinates (N being the number of atoms constituting the molecule), to which are associated that many collective motions. Each one of these motions is individuated by an "eigenvector", that is a motion direction, whose width is expressed by the "eigenvalue"; if the eigenvalues ensemble is sorted in decreasing order, it comes out that only the first n eigenvalues (with $n \ll N$) resemble the largest domain motions.
Such tool could be useful to better clarify whether MW could affect protein dynamics, on the basis of the experimental observation. Although, at the state-of-art, it is not implemented an algorithm which can take into account an external space-time-variable electromagnetic perturbation, could be interesting to characterize the protein dynamics in terms of "principal" modes. Each one of these modes is in fact characterized by its own frequency, which could exhibit resonances in the range of the electromagnetic fields typical of mobile communication. In particular, when globular proteins are in an acidic environment, the polipeptide chains are intrinsically more flexible than in physiological conditions, and so their principal modes may present a larger differentiation. Moreover, it has been showed that electromagnetic fields effects on biological structures probably infer structures non completely formed rather than well-defined architectures.
On the other hand, non-native states of proteins are difficult to describe in a detailed manner with spectroscopic methods because of their dynamical properties; also the structural modeling is harder because of the high dimensionality of their conformational space. In fact, conventional methods of MD do not allow to obtain, at room temperature, significantly denatured structures in reasonable computation times, though considering the protonation of aminoacidic residues reached in an acidic pH environment, which experimentally produce denatured structures.
Moreover, even after having reached in a MD a reasonable denaturation state, there is the further challenge of adequately sampling the conformer population expressed by the protein in such non-native conditions. In fact, in a partially denatured state as the *molten globule* of apomyoglobin, significant torsional and rotational barriers exist between the different compact states, that lengthen the interconversion times between conformers up to millisecond scale. In such instance, conventional MD techniques, which span time ranges of nanoseconds, do not appear suitable.
To overcome such difficulties, has been developed a variety of approaches, including the introduction of perturbative terms into the algorithms or an implicit solvent treatment. Another approach consists in providing additional energy to the molecule by means of thermal "heating" in order to fill the energy gaps between conformers. In practice, the system temperature is temporary raised of some hundred

degrees, and then brought back to basal physiological temperature: in such way, the structure partially unfolds during heating, resembling a molten globule conformer, and then remains "entrapped" in that confomer state once the temperature in brought back to initial level. Repeating the heating processes a number of times with different starting conditions, it is possible to obtain an ensemble of partially denatured structures which mimics the molten globule state.
A preliminary MD simulation of 10 ns has been therefore effected onto the apomyoglobin structure to allow relaxing from the constrained crystal structure (PDB entry: 1MYT). As can be seen in left panel of figure 3, after the first nanosecond the overall deviation from the initial structure does not increase nor oscillate significantly, indicating that the structure is already fluctuating around a stable conformation. This simulation has been compared with an analogous one effected on olomyoglobin, in order to confirm spectroscopic data present in literature [63], specifically that structure and conformational dynamics of apomyoglobin does not differ significantly from its heme-carrying form. In particular, secondary structure content and three-dimensional conformation are mostly conserved.
The output structure of this 10 ns MD simulation of apomyoglobin, has become the starting structure of the cited "heat-driven" unfolding procedure, taking into account the different protonation state expected at very low pH: this has been realized by means of "H++" web server, which provides an algorithm able to calculate aminoacids pKa at any given pH [64]. The heat-driven procedure has been carried out 25 times on the same starting structure, generating 25 different forms of the acidic state. In right panel of figure 3 is represented the root mean square deviation along the whole process: is evident that during the first nanosecond, in which takes place the "heating", the RMSD increases continuously and in disordered manner, while in the range 1–2 ns the structure appears to oscillate smoothly around an equilibrium state.
During the "heating" process, as expected, occurs a loss of ordered structure with occasional appearing of small β-sheet segments. This α and β content is present, at similar extent, in each of the 25 trajectories, and when averaged reproduce the experimental results concerning the acidic state of apomyoglobin. In table 3 are summarized the main structural data obtained from these trajectories, compared to the same data obtained from a free 300 K apomyoglobin trajectory. For these reasons, we consider the trajectories ensemble, though rather small compared to the variety of acidic forms which can be taken by the protein, sufficiently representative of the acidic state of apomyoglobin. It has also to be noticed that the dipole momentum increases up to five-fold respect to the native value, as expected due to residues charging. Snapshots of each of the 25 structures obtained by the cited procedure are represented, in the "cylinder style", in figure 4: though different, is evident the secondary structure content in each of these putative acidic forms.
Once obtained an ensemble of apomyoglobin acidic state, we have analyzed the conformational dynamics of each element of such set by means of the cited Normal Modes Analysis computational technique. Application of such technique has allowed to separate the slow, correlated motions between specific protein districts from the much more numerous and fast atoms fluctuations around their equilibrium positions. The amplitude of such motions is expressed by the eigenvalues calculated by diagonalizing the hessian matrix of the forces, usually in length units. By means of the relationship:

$$\nu_i = 2\pi\sqrt{\frac{kT}{\lambda_i}}\ ,$$

in which k is the Boltzmann's constant and T the absolute temperature, amplitude λ_i of the i-th eigenvector is converted into its relative frequency ν_i [62], to be expressed either in wavenumber (cm^{-1}) or hertz units; is evident that the wider is the motion, the lower is its frequency.
Results of such analysis are showed in figure 5, which include the first 20 eigenvalues, expressed in frequency units, obtained by NMA performed on native apomyoglobin and all 25 acidic forms; first 6 eigenvalues are not shown because, due to removal of overall translational and rotational degrees od freedom, they have zero value. It could be seen that at least for lower eigenvalues indexes (that is lower frequencies), native apomyoglobin motions occur always at frequencies higher compared to acidic forms. However, these frequencies reach values up to tenths or even hundreds of GHz, that is rather far from the range of mobile communication microwaves (which is about few GHz).
Subsequently, we have considered that (1), one of the effect of the pH lowering on a protein solution is an increase of the aggregation rate, due to the solvent exposure of hydrophobic regions caused by partial unfolding; and (2), our observations on apomyoglobin molecular dynamics, either in its native and acidic state, suggest that the "wider" is the structure (as occurs in partially unfolded states), the

larger are its eigenvalues amplitudes (and the lower are the frequencies).
These considerations have pushed us to focus our analysis on possible aggregate forms, built up starting from one of the acidic forms we have obtained. At low pH, in conditions of partial unfolding, globular proteins expose hydrophobic segments of the structure which, in native conditions, pack against each other to form the so-called hydrophobic core of the given protein. Exposed segments of different molecules may, in turn, find energetically favourable pack against each other to form supersecondary structures, or aggregates, in ordered (such as *β-amyloid fibrils*) or disordered manner. To simulate this process we have selected from the 25-acidic-forms ensemble the one which had the most of its hydrophobic residues exposed to solvent, by taking into account both the solvent accessible surface and the absolute Kite-Doolittle hydrophobicity scale [66]. Starting from this chosen form, we have constructed aggregates up to 32 molecules, in order to allow the best trade-off between larger contact areas between hydrophobic residues and electrostatic repulsion; snapshots of the chosen form and its tetramer, 16-mer and 32-mer are depicted in figure 6.
Because reliable energy minimization of such large molecules is beyond available computational capabilities, to analyze collective motions of such aggregates we have followed a strategy which is different from classical molecular dynamics, based fundamentally on newtonian mechanics. In fact, an alternative and faster way to explore the conformational space around a known structure is based on generating new structures taking into account geometric restrictions, internal constraints and other configurational barriers that restrict protein dynamics to a limited number of collective degrees of freedom. One of the available software packages which are based on such non-newtonian method is CONCOORD [67]. The ensemble of structures generated by CONCOORD has been demonstrated to span the same configurational space of an output trajectory of a classical molecular dynamics simulation [67]. Extraction of larger, collective motions which dominate protein conformational fluctuations may be performed by analysis techniques like *Essential Dynamics* (ED)[68]. The method consists of diagonalization of the covariance matrix $\mathbf{C}$ of atomic fluctuations, after removal of overall translation and rotation:

$$C_{ij} = \langle (x_i - \langle x_i \rangle)(x_j - \langle x_j \rangle) \rangle$$

where x are the cartesian atomic coordinates. Resulting eigenvectors are directions in configurational space of which the corresponding eigenvalues give the mean square fluctuation of the displacement in each direction. ED analysis can be applied to any (sub)set of coordinates of the studied molecular system; however, only C_α atoms were included in ED analyses presented here because it has been shown [68–70] that this approach best detects the large-scale concerted motions in proteins. The ED analysis technique is quite similar to NMA in detecting large, concerted motions in protein macromolecules; in particular, provided a well minimized starting structure, they allow to span the same configurational space and so individuate the same collective motions [62].
First three eigenvalue frequencies have been plotted against aggregate size (that is, number of monomeric units), as showed in figure 7. The process trend is evident, and sustain our hypothesis: the larger is the aggregate, the lower is the frequency, and for an aggregate made of 32 molecules (our computational limit) the frequency of 1^{st} eigenvalue is about 4 GHz, quite closer to the frequency range adopted in radiomobile communication. So, a landscape of resonances between microwave electromagnetic fields and dynamics of protein aggregates from acidic conditions could not appear so remote.

6 Conclusions

In our long-termed and wide-featured investigations about non-thermal effects of microwave electromagnetic fields on a protein chosen as a model, the myoglobin from *Thunnus thynnus*, we have collected numerous experimental observations. Spectroscopic measurements carried out on samples exposed and not exposed to perturbing field, which have ranged from molecular absorption to circular dichroism to frequency-domain fluorometry, do not have revealed any significant change on protein 3-D organization, or on its functional ability to bind a specific ligand like CO.
In order to check for possible effects of radiofrequency fields on the structuring process of the protein matrix, that is on the folding process, we have designed experiments putting molecules in an acidic environment, in which myoglobin preserves some secondary structure elements while tertiary structure is almost completely destroyed, in particular in the surroundings of the eminic site. It is supposed that

similar architectures are present in the initial stages of the folding process. From a dynamical point of view, the protein fluctuate among conformational substates almost iso-energetical which interconvert each other, and which are much more numerous compared to native state; moreover, the increase of localized electrostatic charge due to aminoacidic residue protonation may translate into a higher susceptibility of the the protein to interact with an external electromagnetic field.
In such condition of increased flexibility, we have evaluated the possibility that electromagnetic fields exposure may influence in an athermal fashion the reconstitution kinetics of the aminic site, realized raising the pH to its physiological value. Our measurements effectively confirmed this hypothesis, and are supported by the analysis of the tryptophanilic fluorescence lifetime, which showed a narrower distribution if compared to not perturbed conditions. These data have suggested that, in acidic conditions, microwave exposure lowers some energetic barriers, promoting the selective filling of some substates. Interconversions between substates is therefore made harder (or, in statistical terms, less likely) once the perturbation has removed and the barriers raise up to their native level, even due to the progress of accompanying aggregation processes.
Therefore, we have tried to frame these experimental observation in a rigorous mechanical-dynamical scheme by means of molecular dynamics simulations, which gave us an atomic detail level characterization of a sample of apomyoglobin acidic structures. In particular, normal mode analysis performed on all those structures has evidenced vibrational modes at frequencies lower than native state, albeit still far from the range utilized by mobile communication.
We have then taken into account of the aggregation phenomena by modelling small aggregates of acidic apomyoglobin and analyzing vibrational frequencies of these aggregates. As a clear trend, a decrease of the lowest vibrational frequencies with increasing aggregate size has emerged, localizing the window in the range of only few GHz, very close to the frequencies adopted by radiocommunication. This result, in particular, gives an important theoretical argument for ascribing to microwave electromagnetic fields a direct coupling with conformational dynamics of proteins.
Our research, therefore, suggests significant clues about understanding the basic interactions between electromagnetic fields and fundamental biological systems like proteins, at the least for the model we have selected, which however may reasonably be considered a representative for all proteins.

Acknowledgements

References

1. Repacholi, M.H. 2001, Toxicol. Lett. 120, 323.
2. Hyland, G.J. 2000, Lancet 356, 1833.
3. Goldsmith, J.R. 1995, Int. J. Occup. Environ. Health 1, 47.
4. Rothman, K.J. 2000, Lancet, 356, 1837.
5. Kalns, J., Ryan, K.L., Mason, P.A., Bruno, J.G., Gooden, R., and Kiel, J.L. 2000, Shock, 13, 52.
6. Porcelli, M., Cacciapuoti, G., Fusco, S., Massa, R., d'Ambrosio, G., Bertoldo, C., De Rosa, M., and Zappia, V. 1997, FEBS Lett., 402, 102.
7. La Cara, F., Scarfi, M.R., D'Auria, S., Massa, R., d'Ambrosio, G., Franceschetti, G., Rossi, M., and De Rosa, M. 1999, Bioelettromagnetics, 20, 3172.
8. Alekseev, S.I., and Ziskin, M.C. 1995, Bioelectromagnetics 16, 2124.
9. Malyapa, R.S., Aherm, E.W., and Bi, C. 1998, Radiat. Res. 149, 637.
10. Youbicier-Simo, B.J., and Bastide, M. 2000, Radiat. Protect., 1, 218.
11. Zook, B.C., and Simmens, S.J. 2001, Radiat. Res., 155, 572.
12. De Pomerai, D., Daniells, C., David, H., Allan, J., Duce, I., Mutwakil, M., Thomas, D., Sewell, P., Tattersall, J., Jones, D., and Candido, P. 2000, Nature, 405, 417.
13. French, P.W., Penny, R., Laurence, J.A., and McKenzie, D.R. 2000, Differentiation, 67, 93.
14. Leszczynski, D., Joenvaara, S., Reivinen, J., Kuokka, R. 2002, Differentiation, 70, 120.
15. Adey, W.R., Byus, C., Cain, C., Higgins, R,. Jones, R.A., Kean, C.J., Kuster, N., MacMurray, A., Stagg, R.B., and Zimmerman, G. 2000, Cancer Res., 60, 1857.
16. Adey, W.R., Byus, C., Cain, C., Higgins, R., Jones, R.A., Kean, C.J., Kuster, N., MacMurray, A., Stagg, R.B., Zimmerman, G., Phillips, J.L., and Haggren, W. 1999, Radiat Res., 152, 293.
17. Repacholi, M.H., Basten, A., Geski, V., Noonan, D., Finnie, J., and Bath, M.L. 1997, Radiat Res., 147, 631.
18. Utteridge, T.D,. Gebsky, V., Finnie, J.W., Vernon-Roberts, B., and Kuchel, T.R. 2002, Radiat Res. 158, 357.
19. Lai, H., Horita, A., and Guy, A.W. 1994, Bioelectromagnetics, 15, 95.
20. Cobb, B.L., and Adair, E.R. 2002, Bioelectromagnetics Society Annual Meeting. Toronto 2002, abstract book, pp. 24–25.
21. Laurence, J.A., French, P.W., Lidner, R.A., and McKenzie, D.R. 2000, J. Theor. Biol., 206, 291.
22. Adair, R. 2002, Biophys. J. 82, 1147.
23. Bohr, H. and Bohr, J. 2000, Phys. Rev. E Stat. Phys. Plasmas Fluids Relat. Interdiscip. Topics, 61, 4310.
24. Bohr, J., Bohr, H., and Brunak, S. 1997, Biophys. Chem., 63, 97.
25. Bohr, J., and Bohr, H. 1997, Z. Phys. D: At., Mol., Clusters, 40, 186.
26. Copty, A., Sakran, F., Popov, O., Ziblat, R., Danieli, T., Golosovsky, M., and Davidov, D. 2005, Synthetic Metals, 155, 422.
27. Dawkins, A.W.J., Nightingale, N.R.V., South, G.P., Sheppard, R.J., and Grant, E.H. 1979, Phys. Med. Biol., 24, 1168.
28. Weissenborn, R., Diederichs, K., Welte, W., Maret, G., and Gisler, T. 2005, Acta Cryst. D61, 163.
29. Frauenfelder, H., Parak, F., and Young, R. 1988, Annu. Rev. Biophys. Biophys. Chem., 17, 451.
30. Fayer, D.M., 2001, Annu. Rev. Phys. Chem., 17, 451.
31. Bismuto, E., and Irace, G. 1994, J. Mol. Biol. 241, 103.
32. Bismuto, E., and Irace, G. 2001, FEBS Lett., 509, 476.
33. Bismuto, E., Gratton, E., and Lamb, D. 2001, Biophys. J., 81, 3510.
34. Giardina, B., and Amiconi, G. 1981, Methods Enzymol. 76, 57.
35. Alcalà, R., Gratton, E., and Prendergast, F. 1987, Biophys. J., 51, 597.
36. Alcalà, R., Gratton, E., and Prendergast, F. 1987, Biophys. J., 51, 925.
37. Creighton, T.E. 1993. Proteins: structures and molecular properties, W.H. Freeman & Co., New York.
38. Bismuto, E., Mancinelli, F., d'Ambrosio, G., and Massa, R. 2003, Eur. Biophys. J., 32, 628.
39. Yang, Y.T. 1986, Methods Enzymol., 130, 218.
40. Strickland, E.H. 1974, CRC Crit. Rev. Biochem., 113, 175.
41. Ming-Chu, H., and Woody, R.W. 1971, J. Am. Chem. Soc., 93, 3515.
42. Lakowicz, J.R., Gratton, E., Laczko, G., Cherek, H., and Limkeman, M. 1984, Biophys. J., 46, 463.
43. Beechem, J.M. 1992, Methods Enzymol., 210, 37.
44. Creighton, T.E. 1997, Trends Biochem. Sci., 22, 6.
45. Pfeil, W. 1998, Proteins, 30, 43.
46. Barrick, D., and Baldwin, R.L. 1993, Protein Sci., 2, 869.
47. Bismuto, E., Gratton, E., Sirangelo, I., and Irace, G. 1993, Eur. J. Biochem., 218, 213.
48. Fink, A. 1995, Annu. Rev. Biophys. Biomol. Struc., 29, 213.
49. Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., and Dobson, C.M. 1999, Proc. Natl. Acad. Sci. USA, 96, 3590.
50. Dobson, C.M. 1999, Trends Biochem. Sci., 24, 329.
51. Prusiner, S.B. 1998, Proc. Natl. Acad. Sci. USA, 95, 13363.
52. Subbiah, S. 1996, Protein motions. Landes Company, Austin.
53. Mancinelli, F., Caraglia, M., Abbruzzese, A., d'Ambrosio, G., Massa, R., and Bismuto, E. 2004, J. Cell. Biochem., 93, 188.
54. Colonna, G., Irace, G., Bismuto, E., Servillo, L., and Balestrieri, C. 1983, Comp. Biochem. Physiol., 76A, 481.

55. Sirangelo, I., Malmo, C., Casillo, M., Mezzogiorno, A., Papa, M., and Irace, G. 2002, J. Biol. Chem. 277, 45887.
56. Bevington, P.R., and Robinson, D.K. 2003, Data reduction and error analysis for the physical sciences (3^{rd} edition), McGraw-Hill, New York.
57. Eliezer, D., Yao, J., Dyson, H.J., and Wright, P.E. 1998, Nat. Struct. Biol., 5, 148.
58. Gruebele, M. 1999, Annu. Rev. Phys. Chem., 50, 485.
59. Englander, S.W. 2000, Annu. Rev. Biophys. Biomol. Struct., 29, 213.
60. Brooks, B., and Karplus, M. 1983, Proc. Nat. Acad. Sci. USA, 80, 6571.
61. Levitt, M., Sander, C., and Stern, P.S. 1984, J. Mol. Biol., 181, 423.
62. Hayward, S., and Gō, N. 1995, Annu. Rev. Phys. Chem., 46, 223.
63. Bismuto, E., Colonna, G., Savy, F. and Irace, G. 1985, Int. J. Pept. Protein Res., 26, 195.
64. Gordon, J.C., Myers, J.B., Folta, T., Shoja, V., Heath, L.S., and Onufriev, A. 2005, Nucleic Acids Res., 33W, 368.
65. Kabsch, W:, and Sander, C. 1983, Biopolymers, 22, 2577.
66. Kyte, J., and Doolittle, R.F. 1982, J. Mol. Biol. 157, 105.
67. de Groot, B.L., van Aalten, D.M.F., Scheek, R.M., Amadei, A., Vriend, G., and Berendsen, H.J.C. 1997, Proteins, 29, 240.
68. Amadei, A., Linssen, A.B.M., and Berendsen, H.J.C. 1993, Proteins, 17, 412.
69. van Aalten, D.M., Findlay, J.B., Amadei, A., and Berendsen, H.J.C. 1995, Protein Eng., 8, 1129.
70. van Aalten, D.M., de Groot, B.L., Berendsen, H.J.C., Findlay, J.B.C., and Amadei, A. 1997, J. Comp. Chem, 18, 169.

Figure legends

Fig. 1 Comparison between time courses of heme refolding from pH 3.0 to neutral conditions of exposed (dashed) and non-exposed (solid) samples. After exposure, time course were run immediately (0 h), after 4 h and after 8 h, as indicated.

Fig. 2 Comparison between tryptophanil fluorescence lifetime distribution (excitation at 290 nm) having lorentzian shape of exposed and non-exposed samples of acidic tuna apomyoglobin.

Fig. 3 Root Mean Square Displacement from the crystallographic coordinates of a *Thunnus thynnus* apomyoglobin molecule in water, along a free 10 ns MD simulation (left panel) and along a 1 ns MD at 300 K + 1 ns 500 K at acidic pH (right panel).

Fig. 4 Cylinder-style representation of the 25 acidic forms of apomyglobin, as obtained by MD. The colors indentify different α-helix segments, as indentified in the native state, along the sequence, shown in the lower panel.

Fig. 5 Frequency of first 20 modes calculated by Normal mode Analysis carried out on native apomyoglobin (black) and 25 acidic structures (color).

Fig. 6 Aggregates of 4, 16 and 32 acidic apomyoglobin molecules, constructed starting from one acidic form (upper left corner).

Fig. 7 Frequency of first 3 modes of acidic apomyoglobin aggregates, against aggregate size.

Tables

Lifetime lorentzian bimodal distribution		
	Exposed	Not exposed
Center 1	2.67	2.65
Width 1	0.27	0.29
Fraction 1	0.97	0.95
Center 2	0.00	0.00
Width 2	0.05	0.05
χ^2	1.41	1.38

Table 1 Comparison between lifetime lorentzian distribution analysis of tryptophan emission decay of exposed and not exposed apomyoglobin.

Model: $\boldsymbol{y = y_0 + A_1(1 - e^{-t/t_1}) + A_2(1 - e^{-t/t_2})}$

	Exposed		*Non-exposed*	
Parameter	Value	Error	Value	Error
χ^2	2.4831×10^{-6}		2.0886×10^{-6}	
y_0	1.01648	$\pm$ 0.00042	1.01947	$\pm$ 0.00037
A_1	0.37989	$\pm$ 0.00109	0.39116	$\pm$ 0.00126
t_1	143.62115	$\pm$ 0.60453	162.98257	$\pm$ 0.67731
A_2	0.64686	$\pm$ 0.00124	0.74327	$\pm$ 0.00132
t_2	1440.42956	$\pm$ 11.28342	1490.46104	$\pm$ 11.37231

Table 2 Parameters obtained from best-fitting the data relative to 15 pairs of refolding kinetics under reported model.

MD Structural data for native and acidic state						
	Net charge	**R_g**	**α-helix (%)**	**β-sheet (%)**	**SAS (nm^2)**	**M_{tot} (D)**
Native	1.42	+3	69.4	0.0	75.4	1281
Acidic	1.79	+16.6	31.0	1.7	83.2	5674

Table 3 Columns are occupied by net charge overall the sequence, radius of gyration, percentage of alpha and beta content, solvent accessible surface, total dipole momentum. For acidic state, values are averages over all 25 structures.