Eur Biophys J (2003) 32: 628–634
DOI 10.1007/s00249-003-0310-2

ARTICLE

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Are the conformational dynamics and the ligand binding properties of myoglobin affected by exposure to microwave radiation?

Received: 12 December 2002 / Revised: 19 March 2003 / Accepted: 8 April 2003 / Published online: 13 June 2003

Abstract The global uptake of mobile communication emphasizes the question about possible adverse consequences of the exposure to low-level radiofrequency radiation from mobile phones on human health as result of so-called "non-thermal effects". In order to state safety guidelines it seems appropriate to start by excluding, if possible, non-specific effects on structural and dynamic properties of fundamental biomolecules such as proteins. Proteins are flexible polyelectrolytes; thus, they are susceptible, in principle, to the action of electromagnetic fields. In this article, we investigated the effects of microwaves on structural and functional properties of *Tunnus tynnus* myoglobin at 1.95 GHz, a frequency used by new wireless microwave communication systems. The protein solution was exposed for 2.5 h to 51 mW/g SAR (specific absorption rate) level. Measurements of absorption spectroscopy, circular dichroism and fluorescence emission decay in the frequency domain do not exhibit any influence of the radiation on the native structural state of protein macromolecules.

Keywords Frequency domain fluorometry · Non-thermal microwave effect · Protein conformational dynamics

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Introduction

Nowadays, more than one billion people utilize cellular phones and the base stations constituting the cell network are spread everywhere, even in densely populated areas (Hyland 2000). The development of mobile communication has aroused a deep interest in people and has stimulated wide and often controversial discussions in the scientific community about potential damages induced by exposure to low-level radiation emitted in the microwave (MW) region. These effects are indicated as "non-thermal effects" (Hyland 1998) to distinguish them by heating effects observed at higher power. Epidemiological studies (Goldsmith 1995; Rothman 2000) and a remarkable bulk of experimental work, in vitro and/or in vivo, have recently been performed in searching for correlations among MW exposure and important functional and pathological aspects in humans and other organisms. The observations, performed at various frequencies, have been concerned with irregularities in the embryonal growth (Youbicier-Simo and Bastide 2000), altered cell response to oxidative stress (Kalns et al. 2000; Stagg et al. 2001), DNA damage (Malyapa et al. 1998), modifications in the catalytic efficiency of some enzymes (Porcelli et al. 1997; La Cara et al. 1999), promotion of brain cancer (Zook and Simmens 2001), and variations of ion transport flux through specific membrane channels (Alekseev and Ziskin 1995). The complexity of the investigated biological systems, as well as the variability of the conditions of MW radiation exposure (i.e. frequency, signal modulation, duration of exposure), makes it arduous to perform experiments in a reproducible manner. These considerations could explain contrasting reports about the effects induced by MW fields on the same or similar biological samples. For example, a recent report (Adey et al. 2000) has shown no effects on spontaneous and nitrosourea-induced primary tumors of the central nervous system in Fischer 344 rats, exposed to frequency-modulated MW

fields (836.55 MHz) simulating radiofrequency exposures in the head of hand-held mobile users. These negative findings contrast with a previous study (Adey et al. 1999). 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 (Repacholi et al. 1997; Utteridge et al. 2002), or spatial-working memory deficit in rats exposed to low-level MW radiation (Lai et al. 1994; Cobb and Adair 2002). In the latter couple of experiments, Lai et al. concluded from their results that both cholinergic and opioid systems within the brain are affected by MW radiation, while Cobb and Adair demonstrated that, in the same experimental conditions, there is no evidence that exposure to examined levels of MW radiation causes decrements in the ability of rats to complete a maze task.

Recent reports (Laurence et al. 2000; Adair 2002), starting from theoretical considerations, have given contrasting conclusions on the possibility that biological systems coupled to electromagnetic fields can exhibit classical resonance at MW frequency, thus affecting functional properties. However, biomolecules are complex structures with a large variety of intra- and intermolecular interactions. Moreover, as in the case of proteins, they possess a hierarchical organization of internal motions that can involve few atoms as well as a large segment of the structure.

A renewed interest about the bioeffects of MW radiation has been produced by recent observations showing that prolonged exposure to low-intensity MW fields can induce heat-shock responses, suggesting damage to cellular proteins. The heat-shock proteins could be involved as molecular chaperones to rescue damaged proteins. This effect appears to be non-thermal, suggesting that current exposure limits set for MW equipment may need to be reconsidered (De Pomerai et al. 2000; French et al. 2000; Leszczynski et al. 2002).

In conclusion, a wide experimental study aimed at searching for general effects induced by MW radiations appears to us necessary and urgent, since long-term mobile phone exposure could be health hazardous. The preliminary investigation would begin from the basic macromolecules present in cells, such as proteins. In this article, we have explored the effects of electromagnetic fields at the frequency nowadays operating in the mobile communication range (1.95 GHz) on the spectroscopic properties of myoglobin samples at neutral pH, observed by classical techniques such as absorption spectroscopy, circular dichroism, and by use of more sophisticated fluorescence techniques such as frequency domain fluorometry (Gratton and Limkeman 1983). These spectroscopic observations are very effective in giving information on the structural organization and the conformational dynamics of myoglobin (Bismuto and Irace 1994, 2001; Bismuto et al. 2001). The measurements were performed before and after the exposure of protein samples to the MW field. We have chosen myoglobin (specifically from *Tunnus tynnus*) as a protein model on the basis of the following remarks:

1. Myoglobin was the first protein whose three-dimensional 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 hierarchic level of internal motions, since the very first observation 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 (Frauenfelder et al. 1988; Fayer 2001).
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. Moreover, the presence of a single tryptophan residue in the sequence of myoglobin from *Tunnus tynnus* (Colonna et al. 1983) allows an easier interpretation of the data provided by time-resolved fluorimetry.

The spectroscopic investigations in the absorption region of heme group will show possible effects of the MW field about the heme–apoprotein interaction in native conditions as well as about functional capabilities, monitoring the bond with molecules such as CO (Giardina and Amiconi, 1981).

The conformational dynamics of a given protein are effectively pointed out by the analysis of the intrinsic fluorescence emissive decay of single tryptophan proteins (Alcalà et al. 1987a, 1987b). Usually, they are observed as a quasi-continuous distribution of lifetimes rather than one single lifetime, whose mean value is related to the microenvironment experienced on average by the fluorophore 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.

The results reported in this paper indicate that, at least for the native state of the protein, MW perturbation does not affect the structural organization of the myoglobin macromolecule or its internal dynamics and CO binding affinity.

Materials and methods

Myoglobin purification

The main component of tuna myoglobin was prepared according to the method described elsewhere (Bismuto et al. 1989) from the heart ventricle of *Tunnus tynnus*. The homogeneity of the preparation was controlled by sodium dodecyl sulfate/polyacrylamide gel electrophoresis with 15% gels and 5% stacking gels.

CO–myoglobin complex preparation

To myoglobin, dissolved in 50 mM sodium phosphate (pH 7.00), were added a few crystals of sodium dithionite (final concentration 0.2 mg/mL) to ensure that the oxygen was removed. The excess of dithionite was eliminated by gel filtration chromatography on a column (20 cm×1.5 cm) filled with Sephadex G-25 (Pharmacia, Sweden). Finally, myoglobin solutions were saturated with ultrapure carbon monoxide and then placed in optical cuvettes sealed with soft plastic stoppers.

Apomyoglobin preparation

The heme was removed from myoglobin by the 2-butanone extraction procedure (Teale 1959). The contamination of the apoprotein by myoglobin was assessed spectrophotometrically. In all cases, no significant absorption was observed in the Soret region.

Exposure setup

The sample, 3 mL of solution contained in a polystyrene cuvette, was inserted in a thermostatted waveguide exposure system and exposed for 2.5 h at 30 °C. The experimental setup is sketched in Fig. 1a. The MW signal (1.95 GHz, CW; signal source: Marconi, UK, model 6055C) was amplified by a TWT amplifier (Varian, USA, model VZS 6951 K2 BDEK) and was fed through the coaxial isolator (Rayteon, USA, model ICSM9) and the coaxial bridge (Hewlett Packard, USA, model 776D) into the sample holder (Fig. 1b), a rectangular cavity made of a waveguide (110 mm× 55 mm×500 mm) and two coax-to-waveguide adapters (Scientific Atlanta, USA, model n 11-1-7). A step attenuator (Hewlett Packard, USA, model 8494A) was used for regulating the incident power. MW power measurements were carried out with HP 8481A power sensors and HP 435B power meters: the power incident on the sample-holder cavity, P_i, the reflected power, P_r, and the transmitted power, P_t, were measured. The power lost, P_l, in the sample holder unit (sample included) was evaluated as $P_l = P_i - P_r - P_t$. Owing to their non-perturbative character (without invasive probes), these measurements could be performed during the actual exposure of the biological samples. It is a very well consolidated system (Massa 1988), which allows accurate dosimetry during the exposures. The cuvette (10 mm×10 mm×35 mm) was placed vertically where the incident E field (vertical) is a maximum (Fig. 1b). The specific absorption rate (SAR) was calculated from the measured P_l as P_l/m, m being the mass of the sample. Thus, the average SAR and the standard deviation evaluated over all exposures was 51 ± 1 mW/g. This value can be related to the electric field effective value $E_{eff} = 197$ V/m, as a consequence of the relationship:

$$E = \left(\frac{\rho}{\sigma}\mathrm{SAR}\right)^{1/2} \quad (1)$$

(Chou et al. 1996), in which φ is the sample density (in our experiment, closely approaching the density of water, 1000 kg/m^3) and σ is the sample conductivity that was measured under our experimental conditions and taking into account ohmic losses as well as dielectric losses, equal to 1.32 S/m.

In order to guarantee to the sample holder a stable and known temperature, a thermostatted (25 ± 0.1 °C) water jacket was provided, surrounding the waveguide. The temperature at the beginning of the exposure was 25 ± 0.1 °C. It grew to the steady-state level of 30 ± 0.1 °C in about 30 min; afterwards the exposure continued for an additional 2 h at 30 ± 0.1 °C. The temperature–time evolution was measured during the exposure by means of a fiber optic thermometer (Fisotechnologies, USA, model UMI4).

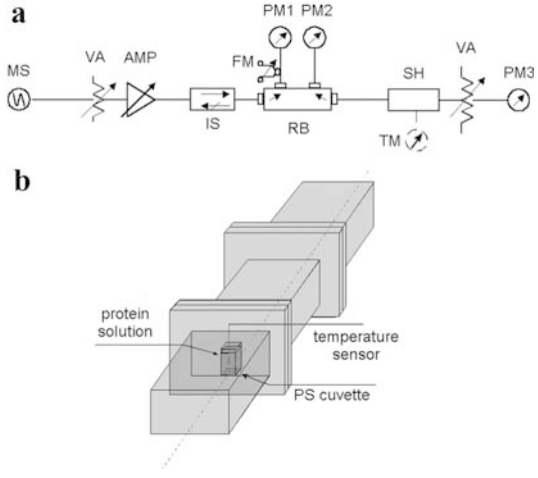

Fig. 1 a The exposure circuit scheme. *MS*, microwave source; *VA*, variable attenuator; *AMP*, amplifier; *IS*, isolator; *FM*, frequency meter; *PM1*, *PM2*, *PM3*, power meters (to measure incident, reflected and transmitted power, respectively); *RB*, 20 dB reflectometer bridge; *SH*, sample holder; *TM*, thermometer. **b** Sketch of the sample holder within the waveguide

CD spectroscopy

Circular dichroism measurements were performed on samples of tuna myoglobin, at protein concentrations of 1.67 μM in 50 mM sodium phosphate at pH 7.0. A spectropolarimeter, model J-710 (Jasco, Japan), equipped with a Neslab RTE-110 temperature controller (Neslab Instruments, USA) and calibrated with a standard solution of (+)-10-camphorsulfonic acid, was used. The cuvettes of 0.1 cm path length (Hellma, USA) were used in the far- (190–240 nm) and 1.0 cm path length in the near-UV (250–330 nm) and visible regions (330–700 nm), respectively. Photomultiplier absorbance did not exceed 600 V in the spectral regions measured. Each spectrum was signal-averaged at least five times, smoothed with Spectropolarimeter System Software, version 1.00 (Jasco), and baseline-corrected by subtracting the buffer spectrum. All the measurements were performed at 30 °C under a nitrogen flow (3 L/h).

Fluorescence emission decay measurements

Frequency-domain techniques were used to measure the fluorescence decay of the apomyoglobin sample in the range 5–200 MHz, using a multifrequency phase shift and modulation cross-correlation fluorometer (GREG 200, ISS, USA). The emission was observed using an optical filter combination of UV 34 and U340 (Oriel, USA); the reference was a glycogen solution as scatterer. The temperature was monitored continuously during measurements by attaching a thermocouple to the sample cuvette. Readings of the thermocouple were monitored by an Omega Digicator (Omega Engineering, USA) with an accuracy of ±0.1 °C. The absorbance of the protein solution did not exceed 0.1 at the exciting wavelength. The lifetime analysis was performed by Global Unlimited (University of Illinois at Urbana) according to Beechem (1992).

Results

Absorbance in the Soret region

The absorption spectrum of tuna myoglobin in the visible region shows (Fig. 2a) a strong band known as the

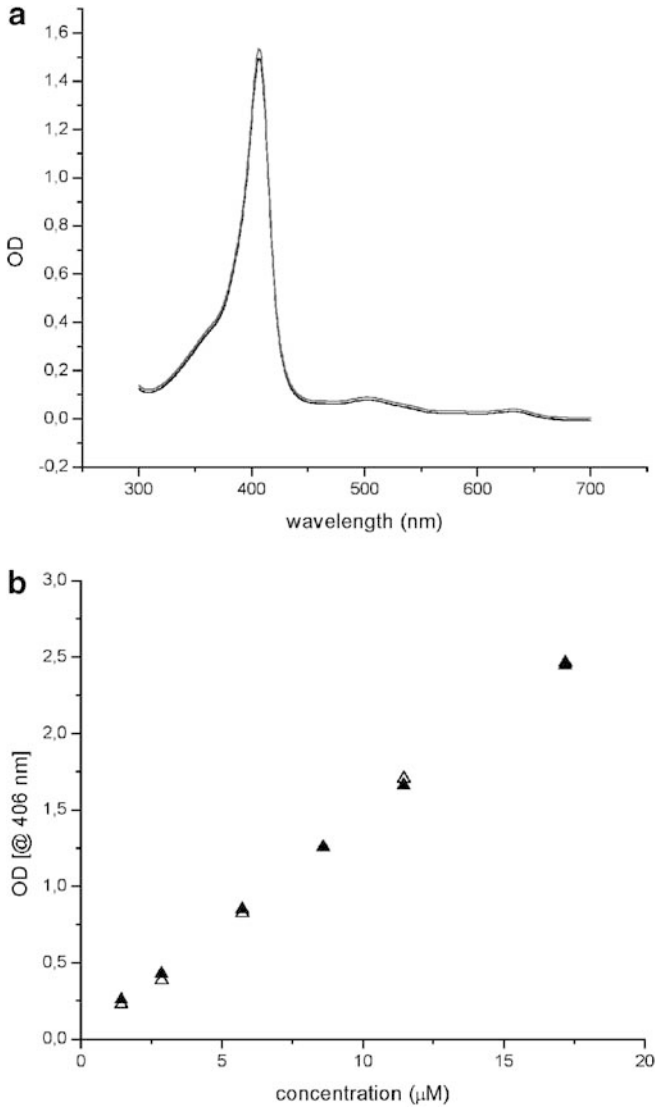

Fig. 2 a Comparison between absorption spectra of exposed (*black*) and not exposed (*grey*) samples, at a protein concentration of 11.45 μM. The temperature of both samples was 30 °C. **b** Comparison between absorption maxima of exposed (*open triangles*) and not exposed (*filled triangles*) samples, against myoglobin concentration. The solvent was sodium phosphate, 50 mM, pH 7.0

Soret region at 406 nm when the iron atom is at the oxidation state III and the sixth ligand position is a water molecule (met-myoglobin). The position and width of such a band are very sensitive to changes in the heme interactions with the surrounding protein. The presence of the MW field did not cause any change in this spectral region, independently from the protein concentration that was variable in the range 1–20 μM (Fig. 2b).

The affinity of myoglobin in the ferrous state for carbon monoxide is about 200 times greater than for oxygen. Because of the CO binding, the main absorption band shifts to 423 nm (Giardina and Amiconi 1981). Figure 3 shows that the exposure experiments on CO–myoglobin samples did not show significant spectral

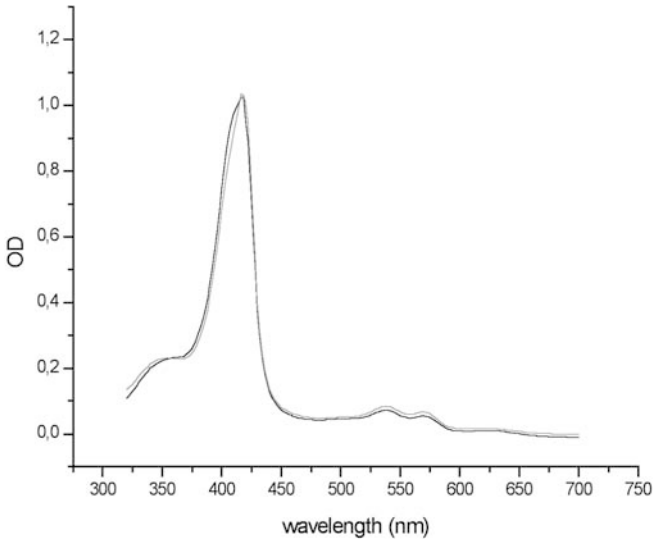

Fig. 3 Comparison between absorption spectra of exposed (*black*) and not exposed (*grey*) samples of CO–myoglobin. The protein solution contained sodium phosphate, 50 mM, pH 7.0. Note the absorption maximum is red-shifted compared to met-myoglobin. The temperature of both samples was 30 °C

changes, thus suggesting that MW radiation does not cause any effect on the binding ligand function of myoglobin.

CD absorption

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 investigation, each containing information concerning a part of the structural organization of the myoglobin molecule:

1. The far-UV region, from 190 to 240 nm. In this region the predominant chromophores are the peptide groups correlated with the secondary structure of the macromolecule, i.e. α-helix, parallel and antiparallel β-sheet, β-turn, and no ordered region content (Yang 1986).
2. The near-UV region, from 240 nm to 300 nm. In this region the predominant chromophores are the aromatic amino acid side chains, which may give detailed information on ''local'' chain–chain interactions (Strickland 1974).
3. The region above 300 nm, including the visible. No amino acid chromophores contribute directly to this region, but CD spectra of the heme transitions are governed by the asymmetry of protein environment.

The optical activity of myoglobin spectra depends on the relative positions of the different chromophores in

the three-dimensional organization of the molecule (Ming-Chu and Woody 1971). Alterations 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 correlate to specific structural aspects and changes induced by the presence of the MW field. Figure 4 shows the CD spectra of tuna myoglobin in the above indicated three regions. The protein samples were subjected to the continuous action of the MW field for 2.5 h with an average SAR of 51 ± 1 mW/g and at 30 °C (black lines). In the same figure are reported the spectra relative to a myoglobin sample not exposed to the MW perturbation at the same temperature (grey lines). The MW radiation does not appear to modify the CD spectra of Fig. 4. Specifically, the examination of the CD spectra in the (a), (b) and (c) parts of Fig. 4 allows us to conclude that the secondary (essentially α-helical) structure, as well as the tertiary structure, at least for the protein regions forming the aromatic amino acidic residue surroundings, are not affected by the MW field in the region 1.8–2.2 GHz. The absence of any change in the Soret absorption band of Fig. 4 indicates that the contacts of the heme with other amino acidic residues, including His64, Phe33 and Tyr103 (Ming-Chu and Woody 1971), are not affected by resonance coupling with the MW field.

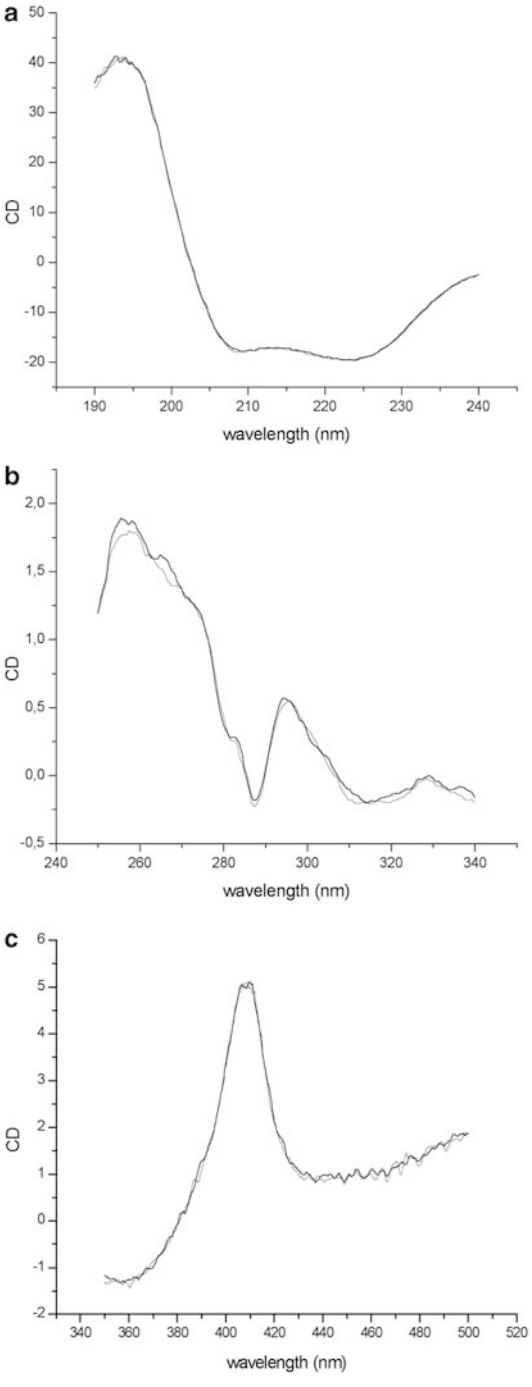

Fig. 4 Comparison between CD spectra of exposed (*black*) and not exposed (*grey*) myoglobin solution samples: **a** far-UV, **b** near-UV and **c** visible. The protein solution contained sodium phosphate, 50 mM, pH 7.0. The dichroic activity is expressed as Δε (M^{-1} cm^{-1}). The temperature of both samples was 30 °C

Frequency domain fluorometry

The tryptophanyl emission decay properties of tuna apomyoglobin, a single tryptophan protein, was investigated by frequency domain fluorometry in the presence and in the absence of the MW field at 1.95 GHz and 30 °C, as already indicated for the CD spectral determinations. The phase shifts and demodulation factors of the intrinsic fluorescence were collected upon excitation at 290 nm using modulation in the frequency range 5–200 MHz. The data were analysed by nonlinear least-squares routines using algorithms for multi-exponential as well as distributional analysis, as reported elsewhere (Lakowicz et al. 1984; Beechem 1992). 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. The structural heterogeneity of a given protein is effectively pointed out by the analysis of the intrinsic fluorescence emissive decay of single tryptophan proteins. Usually, it is observed as a quasi-continuous distribution of lifetimes rather than one single lifetime, whose mean value is related to the microenvironment experienced on average by the fluorophore 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. The analysis reported in Table 1 shows that the emission decay of tuna apomyoglobin, except for a very short-lived component which contributes negligibly to the total fluorescence, and that probably originates from weakly scattered light or other unpredictable instrumental contributions (Barbieri et al. 1989; Bismuto et al. 1993), is a distribution of lifetimes with a center at 2.6 ns and a full width at half maximum of 0.40 ns. The lifetime distribution parameter, both for irradiated and non-irradiated apomyoglobin molecules

Table 1 Comparison between lifetime discrete and lorentzian distribution analysis of tryptophan emission decay of exposed and not exposed apomyoglobin in 50 mM sodium phosphate (pH 7.00) at 30 °C

Tuna myogbobin		Lifetime discrete analysis at neutral pH	
		Exposed	Not exposed
Monoexponential			
	Lifetime	2.50	2.46
	χ^2	60.1	58.0
Biexponential			
	Lifetime 1	2.50	2.46
	Fraction 1	0.97	0.97
	Lifetime 2	0.14	0.14
	χ^2	5.09	5.12
Triexponential			
	Lifetime 1	24.2	24.1
	Fraction 1	0.00	0.00
	Lifetime 2	2.68	2.68
	Fraction 2	0.97	0.97
	Lifetime 3	0.00	0.00
	χ^2	3.60	3.35
		Lifetime lorentzian distribution at neutral pH	
		Exposed	Not exposed
Unimodal			
	Centre	2.57	2.59
	Width	0.68	0.71
	χ^2	12.4	12.2
Bimodal			
	Centre 1	2.67	2.65
	Width 1	0.27	0.29
	Fraction 1	0.97	0.95
	Centre 2	0.00	0.00
	Width 2	0.05	0.05
	χ^2	1.41	1.38

(shown in Table 1), 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.

Discussion

A working molecule of myoglobin can assume a large number of almost isoenergetic subconfromations. Conformational transitions between different forms are the important link between structure and function (Frauenfelder et al. 1988). Nowadays, protein motions are in general believed to be involved in many basic functions such as catalysis, regulation, transportation and aggregation (Subbiah 1996). Myoglobin fluctuations occur around the native architecture that is determined by stabilizing interactions, among which electrostatic forces perform an important role (Crighton 1993). Figure 5b shows the electrostatic potential surface of myoglobin due to the heterogeneity in the charge and polarity of its constituting amino acid residues. This picture allow us to hypothesize that the action of an oscillating electromagnetic field could induce forced vibrations in the protein structure able to affect its conformation. In particular, it has also been calculated that the region surrounding the heme binding site possesses a negative charge density that could be perturbed by an exciting MW field. The perturbation could affect the accessibility of this functional region to water or ligand molecules.

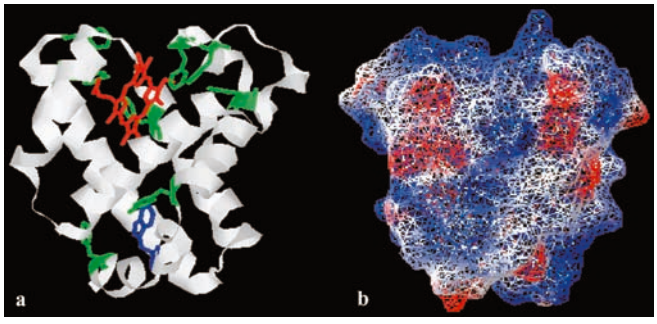

Fig. 5 a Schematic representation of the 3-D structure of *Tunnus tynnus* myoglobin. The heme group is depicted in *red*; the sole Trp residue is colored in *blue*; the other aromatic residues (Phe and Tyr) are indicated in *green*. The protein structure was visualized by RasMol v2.6. **b** Molecular surface electrostatic potential by charged residue coulombic potential, calculated using Swisse-Pdb-Viewer v3.7b2 (Guex and Peitsch 1997). The electric potential increases on going from red to blue. In the calculation, solvent and protein dielectric constants were taken as 80 and 4.0, respectively

In this article, we have explored the action of variable electromagnetic fields of frequencies nowadays operating in mobile communications (1.95 GHz). The possibility to obtain structural, dynamic and functional information by spectroscopic techniques such as absorption spectroscopy, circular dichroism and time-resolved emission decay of intrinsic fluorescence is due to the presence of intramolecular groups shown in Fig. 5a. Thus, the analysis of the spectroscopic results can give structural information on the whole protein as well as on specific regions such as the heme pocket or the α-helix in which the tryptophan residue is located. The comparison of the spectroscopic measurements, performed before and after the exposition of samples to the MW field, indicates that MW perturbation does not affect the structural organization of the myoglobin macromolecule or its internal dynamics and CO binding affinity. At present time we intend to investigate the effect of the MW exposure on myoglobin solutions at acidic pH and other non-native conditions to verify the possibility that the protein folding process could be affected by MW irradiation in a non-thermal way.

Acknowledgements This work was supported by the Italian Ministry of Health (SVE.4-ICSA/RF00-448, 2000). We also thank Dr. Ferdinando Febbraio and Dr. Maurizio Tamburini for help and suggestions.

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