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Non-Thermal Effects of Electromagnetic Fields at Mobile Phone Frequency on the Refolding of an Intracellular Protein: Myoglobin

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Abstract Non-thermal effects induced by exposure to microwave electromagnetic field (MW-EMF) at 1.95 MHz, a frequency used in mobile communication, have been observed on the refolding kinetics of the heme binding site in an intracellular protein: tuna myoglobin, starting from acidic conditions. We have selected myoglobin because it can be considered a good model to study protein interactions with MW-EMF for its well-known high-resolution crystallographic structure. Myoglobin solutions at pH 3.0 were subjected to 3 h exposure to microwave field (with a specific absorption rate of 51 ± 1 mW/g); the heme site refolding has been followed by measuring the molecular absorption in the Soret spectral region and the data were fitted to a bi-exponential model. The kinetics of exposed samples appear to be slowered by MW-EMF action. Moreover, the tryptophanyl lifetime distribution of the exposed protein, as deduced by the analysis of the fluorescence emission decay from its single tryptophan, appears sharper if compared to non-exposed protein samples. This observation suggests that the presence of MW-EMF could affect the propensity of protein molecules to populate specific conformational substates among which myoglobin molecules fluctuate at acidic pH. Changes in the structural fluctuation caused by MW perturbation can affect differently the aggregation process that occurs competitively during the protein folding, so representing a potential risk for protein ''misfolding.'' These data suggest that MW-EMF could have also biochemical and, consequently, biological effects on eukaryotic cells that are still under investigation. J. Cell. Biochem. 93: 188–196, 2004.

Key words: microwaves non-thermal effects; frequency domain fluorometry; protein folding, misfolding; protein conformational dynamics

Mobile phone use has dramatically increased with reducing costs and industrial sources suggest that there will be over one billion users world-wide by 2005 [Repacholi, 2001]. As a consequence there is an increasing public interest about health hazard due to radiofrequency fields exposure [Hyland, 2000; Laurence et al., 2000; Adair, 2002]. Although few epidemiological studies are available about radiofrequency exposure and development of specific pathologies [Goldsmith, 1995; Rothman, 2000], suggestive evidences for a health risk have been presented in the recent literature [Malyapa et al., 1998; French et al., 2000; Kalns et al., 2000; Youbicier-Simo and Bastide, 2000; Higashikubo et al., 2001; Zook and Simmens, 2001; Leszczynski et al., 2002; Mashevich et al., 2003]. Non-thermal effects of microwave exposure at frequencies of mobile phone, i.e., 800–1,800 MHz, have been described in several reports: e.g., 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 [De Pomerai et al., 2000; French et al., 2000;

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Hyland, 2000; Leszczynski et al., 2002]. Several neurodegenerative diseases have been associated with the unfolding process and subsequent fibrillization of proteins. Each protein can aggregate at high enough protein concentration and under suitable external conditions (pH, salt concentration, temperature) and the protein regions that are susceptible to fluctuations can be more susceptible to aggregation processes by nucleation-growth mechanism [Chiti et al., 1999; Dobson, 1999]. The present report investigates possible non-thermal effects of MW on the refolding of acidic myoglobin as model protein. At pH 3.0 this protein possesses a net negative charge distributed along the polypeptide chain [Colonna et al., 1983]. Myoglobin is an intrinsically flexible structure [Frauenfelder et al., 1988; Creighton, 1993] 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 [Subbiah, 1996] 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 [Laurence et al., 2000; Adair, 2002]. Myoglobin can be considered a good model to study protein interactions with MW-EMF because its crystallographic structure is known at high-resolution. Moreover, structural investigations can be appropriately performed by spectroscopic techniques measuring the heme molecular absorption or the intrinsic fluorescence decay arising from the single tryptophan residue for the specific case of *Tunnus tynnus* myoglobin [Bismuto et al., 1983, 2001; Colonna et al., 1983; Bismuto and Irace, 1994]. Tryptophanyl emission decay observed in the frequency domain is a very useful tool for investigating the conformational dynamics of proteins [Alcalà et al., 1987a,b]. In fact, due to its intrinsic flexibility, a protein in solution, even in its native state, fluctuates among a large number of conformational substates differing from each other for some structural details [Frauenfelder et al., 1988]. In each substate the excited tryptophan experiences a specific microenvironment that affects its fluorescence lifetime. Myoglobin molecules fluctuate fast at acidic pH and in the presence of salt among unfolded and partly folded states with characteristics of molten globule, i.e., a native-like architecture but with labile tertiary interactions [Barrick and Baldwin, 1993; Bismuto et al., 1993, 1996; Fink, 1995]. These acidic forms of myoglobin with α-helix content come out during the initial stages of myoglobin refolding, and in the folded state pack against each other with large hydrophobic contact areas forming the heme binding site. In this article, we investigated 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). The results suggest that MW-EMF slowers this process. Moreover, we investigated the effect of MW-EMF on conformational dynamics of acidic myoglobin molecules by examining the intrinsic fluorescence decay of tuna myoglobin at pH 3.0 in exposed and non-exposed samples. The results shows that MW-EMF could affect the spectrum of populated conformational substates at acidic pH, causing a different content of protein molecules irreversibly unfolded.

MATERIALS AND METHODS

Myoglobin Purification

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

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.

Protein Exposure to Microwave Radiation

The experimental setup is sketched in Figure 1. The microwave signal (1.95 GHz, CW; signal source: MARCONI INSTRUMENTS 6055C, 0.8–2 GHz), was amplified by a TWT amplifier (VARIAN VZS 6951 KZBDEK) and was fed through the coaxial isolator (RAYTEON

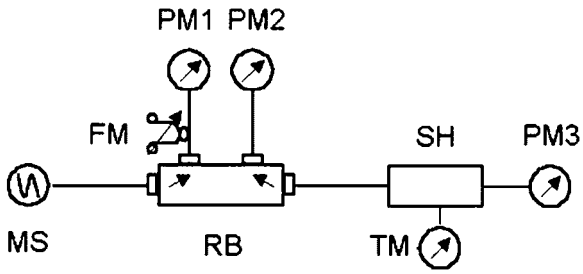

Fig. 1. Semplified exposure circuit scheme. MS, microwave source; FM, frequency meter; PM1, PM2, PM3, power meters (to measure incident, reflected, and transmitted power, respectively); RB, 20 dB reflectometer bridge; SH, sample holder; TM, thermometer.

ICSM9) and the coaxial bridge (HP 776D) into the sample-holder, a cavity made of a rectangular waveguide ($110 \times 55 \times 500$ mm) and two coax-to-waveguide adapters (SCIENTIFIC ATLANTA Mod. N 11-1-7). A step attenuator (HP 8495A) 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 P_i, incident on the sample-holder cavity, the reflected one P_r, and the transmitted one P_t, were measured. By assuming a negligible power loss in the metal walls of the cavity, the power absorbed by the sample P_a was evaluated as $P_a = P_i - P_r - P_t$. The sample, 3 ml of solution contained in a polystyrene cuvette ($10 \times 10 \times 35$ mm) was placed vertical where the incident E field (vertical) is maximum. A thermostated ($25 \pm 0.1^\circ$C) water jacket was provided, surrounding the waveguide. The sample temperature at the beginning of the exposure was $25 \pm 0.1^\circ$C and grew up to the steady state level of $30 \pm 0.1^\circ$C in about 30 min; afterwards the exposure continued for additional 2.5 h at $30 \pm 0.1^\circ$C. The temperature time course was measured during the exposure by means of a fiber optic thermometer (FISOTECHNOLOGIES UMI4). The constance of temperature throughout the whole sample volume was assured by measures at different points of the sample. For the sake of providing a "sham" exposure, a cuvette containing an equal volume of the sample was placed into a thermal bath along the exposure duration for each experiment. Moreover, in these "sham" conditions, we reproduced the same temperature time course of the MW-EMF exposed sample. The average specific absorption rate (SAR) was calculated from the measured P_a as P_a/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. The average SAR, the SAR standard deviation, and the SAR relative dispersion ($RD = \sigma_{SAR}/SAR$), were also calculated, as described elsewhere [Bismuto et al., 2003].

Refolding Experiments of Heme Pocket

The myoglobin refolding from acidic conditions was investigated by fast mixing of 400 μl of 100 mM sodium phosphate, pH 7.0 in 2 ml of acidic myoglobin (50 mM sodium phosphate, pH 3.0). The temperature was carefully controlled and its value was 30°C in each experiment. The absorption at 406 nm was recorded 2 s after mixing. Both exposed and non-exposed acidic myoglobin samples were subjected to refolding procedure, all samples being run blind to avoid bias of results: the experimenter did not know which of the samples was exposed or not (the sham) until the entire batch was processed. The assignment of samples and the exposure/non-exposure criteria was chosen randomly. The fit of kinetic data analysis and statistical tests were performed by arranging appropriate routines in C language [Press et al., 1989].

Fluorescence Emission Decay Measurements

Frequency-domain techniques were used to measure the fluorescence decay of myoglobin samples at pH 3.0 in the range 5–200 MHz, using a multi frequency phase shift and modulation cross-correlation fluorometer GREG 200 (ISS) [Gratton and Limkeman, 1983]. The emission was observed using an optical filter combination of UV 34 and U340 (ORIEL CORP.), 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) with an accuracy of $\pm 0.1^\circ$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 Studies

The absorption spectrum of tuna myoglobin in the visible region shows a strong band, the well known Soret region, at 406 nm when the

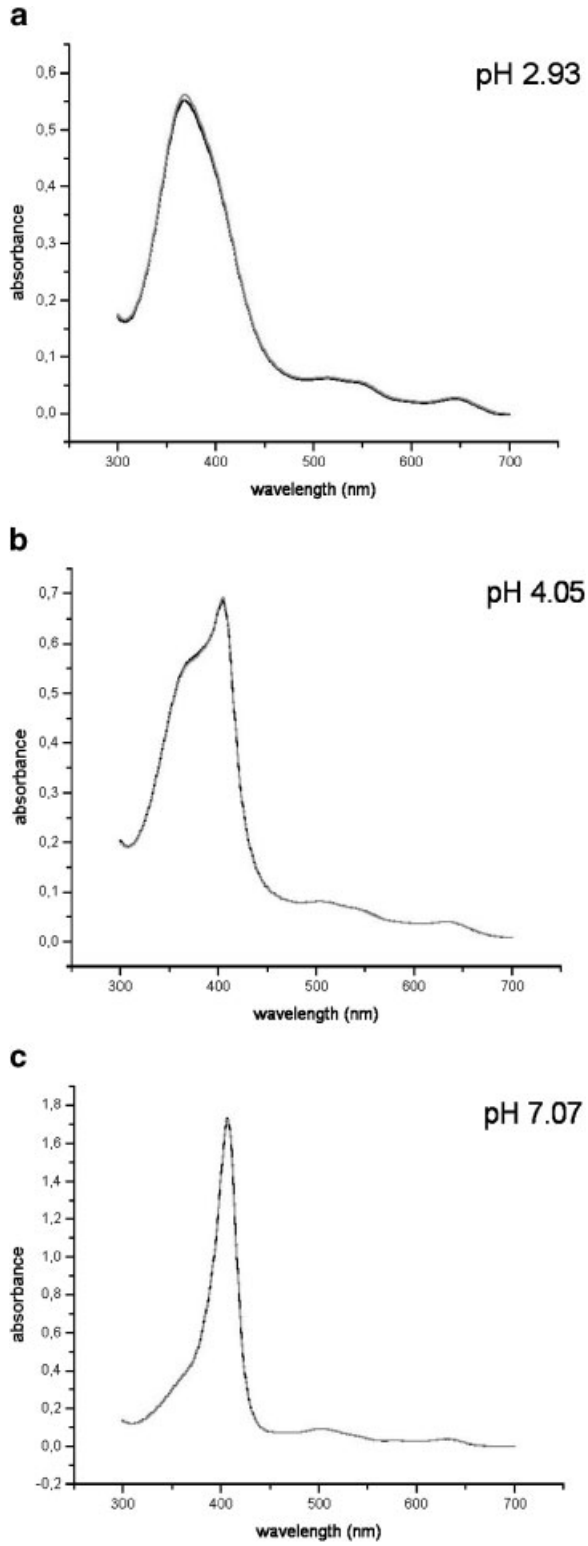

Fig. 2. Comparison between absorption spectra of exposed (black) and non-exposed (grey) samples, at protein concentration 11.45 μM at the indicated pH. The temperature of both samples was 30°C. The buffer at each pH was sodium phosphate, 50 mM.

iron atom is at the oxidation state III and the sixth ligand position is a water molecule (met-myoglobin), as shown in Figure 2c. The position and the width of such band are very sensitive to changes in the heme interactions with the surrounding protein. The lowering of pH causes the destruction of the hydrophobic binding site of the heme [Bismuto et al., 1983]. The release of the prosthetic group in the solvent has as a consequence the spectral blue shift of the Soret to 385 nm (Fig. 2a). The middle part of Figure 2, relative to sample protein at pH 4.05, evidences the existence of an equilibrium between a native structural state and the acidic state with the unfolded heme region. The presence of the MW field did not caused any change in this spectral region, independently from the pH value, as shown in Figure 2 itself. The inability to observe effects in the heme absorption could mean that the heme group in the acidic solution or apomyoglobin is completely accessible to the solvent molecules, because the ligand binding site of the protein structure is disrupted. In this situation the action of MW-EMF could affect the interaction between the prostetic group and the solvent molecules but the effect could be masked by thermal fluctuations completely.

Refolding Studies of Heme Pocket

Myoglobin refolding is a multistep process which competes with irreversible events related to protein misfolding and aggregation [Sirangelo et al., 2002]. The irreversibility in the refolding process is time dependent as shown in Figure 3, where the increasing absorption in the Soret region indicates the reconstitution of heme pocket of the native structure of myoglobin; 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. The myoglobin refolding from acidic conditions was investigated as indicated in the "Materials and Methods" section at 30°C both in the presence and in the absence of perturbing MW-EMF. The refolding kinetics appeared to be slower in the exposed sample against the corresponding unperturbed one. Specifically, Figure 4 shows the curve resulted from the average data relative to 15 pairs of refolding kinetics experiments on exposed and not to MW-EMF protein samples. The parameters characterizing the refolding kinetics and obtained by best fitting of the averaged data to a bi-exponential model are

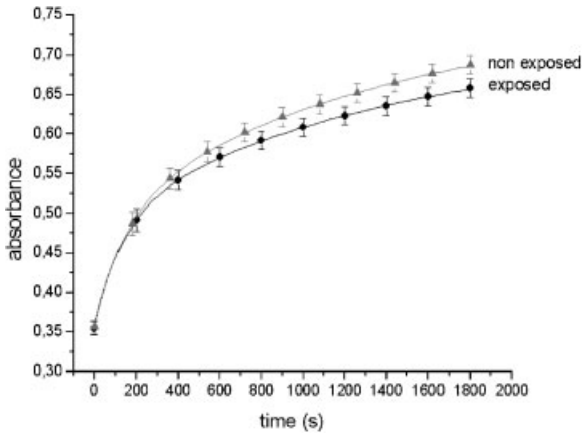

Fig. 3. Fits to bi-exponential model of the average time course of heme refolding from pH 3.0 to neutral conditions (50 mM, sodium phosphate pH 7.0), followed by means of the absorbance at 406 nm, as reported in the ''Experimental'' section: Exposed and non-exposed (sham) tuna myoglobin. The fits were performed on the average data obtained from 15 pairs of kinetic experiments; error bars represent one standard deviation. The temperature was 30°C.

summarized in Table I. Although is difficult to attribute a molecular significance to these parameters, they testimony anyway the slowering of the refolding process caused by the exposure of acidic myoglobin solution to the microwaves action. The statistical significance of the differences in the parameters between the two groups of kinetic curves was assessed by a 10% level F-test on the equality of variance [Press et al., 1989; Bevington and Robinson, 2003]. The application of the Fisher's test by using the data of Table I (i.e., the Chi-square, the degrees of freedom of the bi-exponential model and considering that the absorption data point collected for describing the time course are 900) produces a 1.244 value that is larger than the 1.104 obtained by numerical integration of the Fisher distribution. On the basis of this result, the hypothesis of equal variance could be rejected. Thus, there is a reasonable statistical evidence that the collapsing process of reconstruction of the heme pocket and the binding of heme could be altered by the presence of MW-EMF.

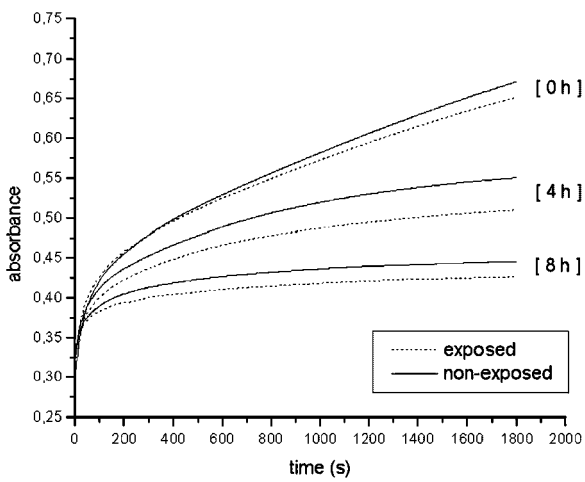

Fig. 4. Comparison between time courses of heme refolding from pH 3.0 to neutral conditions (50 nm, sodium phosphate pH 7.0) of exposed (dashed) and non-exposed (solid) samples. After exposition, time courses were run immediately (0 h), after 4 h and after 8 h, as indicated in the figure. The temperature was hold to 30°C during the whole experiment.

Frequency Domain Fluorometry Studies

The tryptophanyl emission decay properties of tuna myoglobin, a single tryptophan protein, at pH 3.0 was investigated by frequency domain fluorometry in presence and in absence of the MW field at 1.95 GHz and 30°C as already indicated for absorption spectral determinations. At this acidic conditions the disruption of heme binding site occurs and the prosthetic group is released in the solvent or weakly adsorbed to the apoprotein. The heme removal causes the re-appearance of the tryptophanyl fluorescence [Hochstrasser and Negus, 1984; Bismuto et al., 1989] whose emission decay is measured from the phase shifts and demodulation factors collected upon excitation at 290 nm using modulation in the frequency range from 5 to 200 MHz. A set of six independent exposures to MW-EMF for 3 h at 30°C of acidic myoglobin samples were performed and correspondingly frequency domain fluorescence data were collected. For comparison other six determinations were performed on acidic myoglobin samples held at 30°C for the same time but in the absence of MW-EMF. The two data sets were globally analyzed by nonlinear least squares routines using algorithms for multi-exponential as well as distributional analysis by linking the fit parameters 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 uni-modal lifetime distribution on the basis of the chi-square 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 [Alcalà et al., 1987a,b; Bismuto et al., 1988]. Usually, a quasi-continuous distribution of lifetimes is observed rather

TABLE I. Parameters of the Fit Relative to Data of 15 Pairs of Refolding Kinetics Experiments Using the Under Reported Model

	Model: $y = y_0 + A_1(1 - e^{-t/t_1}) + A_2(1 - e^{-t/t_2})$			
	Exposed		Non-exposed	
Parameter	Value	Error	Value	Error
χ^2	2.4831×10^{-6}		2.0886×10^{-6}	
y_0	1.01648	±0.00042	1.01947	±0.00037
A_1	0.37989	±0.00109	0.39116	±0.00126
t_1	143.62115	±0.60453	162.98257	±0.67731
A_2	0.64686	±0.00124	0.74327	±0.00132
t_2	1440.42956	±11.28342	1490.46104	±11.37231

than a single one. The mean value of the lifetime distribution is related to the micro-environment experienced on average by the fluorophore in the excited state inside the protein matrix. The width of 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. The tryptophanyl lifetime parameters are reported in Table II for both irradiated and not irradiated apomyoglobin molecules. Although statistical criteria do not allow to exclude definitively a discrete model to interpretate the emission decay data, a distributional model of fluorescence lifetime appears to be more convincing because of the dynamic nature of the proteins, which are fluctuating among a large number of subconformations [Frauenfelder et al., 1988]. Figure 5 shows the comparison between tryptophan lifetime distribution of both exposed and non exposed acidic myoglobin that appear to be rather different, suggesting that at acidic conditions the spectrum of the conformational substates accessible to apomyoglobin molecules are modified by the MW exposure. Table III reports the 67% χ^2 confidence interval for the distributional parameters of acidic myoglobin solution exposed and non-exposed to MW-EMF. The interval are centered at different values of lifetime but they are rather overlapping.

TABLE II. Comparison Between Lifetime Discrete and Lorentzian Distribution Analysis of Tryptophan Emission Decay of Exposed and Non-Exposed Myoglobin in 50 mM Sodium Phosphate pH 3.00 at 30°C

	Lifetime discrete analysis	
Tuna myoglobin	Exposed	Non-exposed
Monoexponential		
Lifetime	2.36	2.64
Chi-square	74.1	68.0
Biexponential		
Lifetime 1	4.82	5.12
Fraction 1	0.41	0.53
Lifetime 2	1.58	1.18
Fraction 2	0.59	0.47
Chi-square	6.86	8.6
	Lifetime lorentzian distribution	
Unimodal		
Center	2.33	2.10
Width	1.37	2.68
Chi-square	4.0	3.2

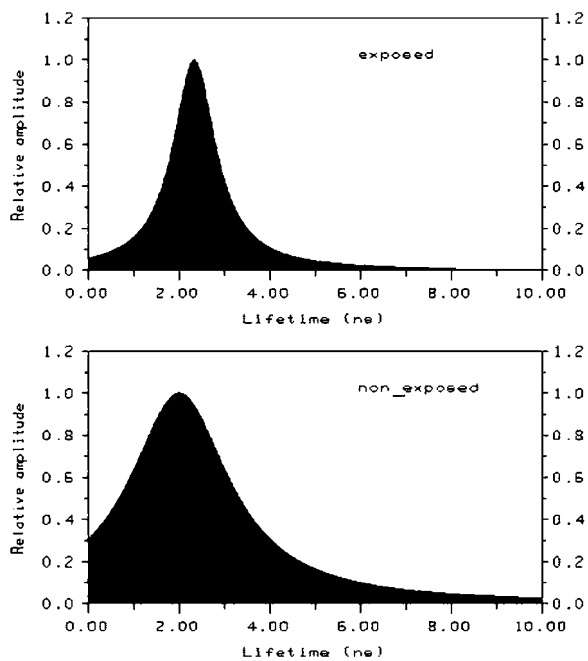

Fig. 5. Tryptophanyl lifetime distribution having lorentzian shape of exposed and non-exposed to MW-EMF samples of acidic tuna myoglobin. The results of six independent experiments performed on different acidified myoglobin samples were analyzed by Global Unlimited [Beechem, 1992]. Excitation was at 290 nm, emission was collected through an optical filter combination of UV34 and U34. Protein concentration was 5 μM and the solution contained 50 mM sodium phosphate, pH 3.0. Temperature was 30°C.

TABLE III. Parameters and Their 67% Confidence Intervals Derived From the Tryptophanyl Emission Decay Analysis in Terms of Unimodal Lorentzian Distribution

	$c_{1\ (ns)}$	$w_{1\ (ns)}$
Exposed	2.33 (+0.096, −0.095)	1.37 (+0.276, −0.269)
Non-exposed	2.10 (+0.089, −0.088)	2.68 (+0.202, −0.200)

DISCUSSION

Structural and functional properties of native proteins could not be affected by the presence of MW-EMF [Adair, 2002]. Recently, it was shown that structural and ligand binding properties of native tuna myoglobin were not perturbed by the presence of MW-EMF [Bismuto et al., 2003]. However, this conclusion could not be true in general, at least when non native protein structural states are considered. The results, here reported, suggest that the microwaves action could affect important biological events like protein 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 [Creighton, 1997; Pfeil, 1998]. Possible coupling resonances to an external MW-EMF of electric charges and dipoles in each protein conformation can change the number of native contacts and consequently the refolding rate or even its capability to be a foldable conformer. The absorption time dependence investigations indicate that MW-EMF alters the refolding kinetics of heme binding site, at least for those events concerning slow adaptation movements of both heme and myoglobin polypeptide chain. The tryptophanyl emission decay experiments performed on tuna myoglobin acidic solutions corroborate our idea that also the conformational dynamics of the partly folded protein could be affected by the action of MW-EMF. Tuna myoglobin contains 146 residues organized into eight strands of mostly α-helical segments, labeled A–H [Colonna et al., 1983]. At low pH and specific salt concentrations, myoglobin forms various destabilized structures (usually indicated as I and E forms) that are variably extended, depending on conditions. Thermodynamic and kinetic studies have shown that the I form contains a tight core consisting of the intersection of the AGH helices and loose solvated helical regions portions of the B–F loop [Barrick and Baldwin, 1993; Fink, 1995; Bismuto et al., 1996]. A species formed at even lower pH and low salt, the E (extended) form, contains essentially only the AGH core and is otherwise very extended [Challender et al., 1998; Baldwin and Rose, 1999a,b]. The dynamics of these partially folded states of apomyoglobin have been characterized at equilibrium using multi-dimensional NMR spectroscopy, fast kinetic investigations by stopped-flow CD and hydrogen exchange kinetics [Eliezer et al., 1998; Gruebele, 1999]. Englander [2000], 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. How often intermediates are productive or unproductive could be affected by the presence of MW-EMF which could decrease some energy barriers. Under weakly folding conditions, the polypeptide fluctuates between these unfolded states exposing different external surfaces. Such surfaces can be differently able to state strong intermolecular interactions, like electrostatic and hydrophobic forces, so that they could cause aggregation phenomena which compete with the refolding process at a different extent. The action of microwave field on the energetic barriers existing in the refolding pathway could explain both the differences observed for tryptophanyl lifetime distribution and the kinetics of heme binding site reconstitution. Our results confirm data recently reported by Bohr and Bohr [2000], relative to the case of β-lactoglobulin. De Pomerai et al. [2003] have also shown that exposure to microwave radiation enhances the aggregation of some proteins, specifically bovine serum albumin, and promotes amyloid fibril formation in bovin insulin. However, the present is the first report on the MW-EMF effects on an intracellular protein suggesting potential consequences on biochemical pathways, still under investigation.

In conclusion, the perturbing action of the MW radiation on acidic myoglobin structure

could also affect the competitive aggregation process that occurs during the protein folding. Thus MW-EMF could represent a potential risk for protein "misfolding" to which can be related several so called "amyloid pathologies" among them prion disease [Prusiner, 1998]. In this sense, it appears interesting a recent report [Sirangelo et al., 2002] concerning a double mutant of sperm whale myoglobin able to form α-amyloid polymeric structures at acidic conditions that confirm our idea of myoglobin as a good model for exploring a potential role of MW-EMF exposure in affecting folding process and/or determining the misfold of unfolded polypeptide chains.

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