Investigation of Potential EMF Induced Conformational Changes of ThermoSensor Protein GrpE of E. coli in Real Time

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Abstract
Previous studies on potential interactions of electromagnetic field (EMF) with proteins have reported that EMFs affect protein structure and folding kinetics. In this study the isolated thermosensor protein GrpE of the Hsp70 chaperone system of E. coli was exposed to EMFs at different frequencies and field strengths under strictly controlled environmental condition. Potential structural changes were monitored with circular dichroism spectroscopy whilst simultaneously recording the temperature at the point of observation.

The specific properties of the protein GrpE allow for distinguishing between thermally and non-thermally induced conformational changes. Since the transition is fully reversible, any direct effects of EMF on the conformation will result in a shift of the equilibrium states. The overall uncertainty of the experimental system was evaluated and based on these results the number of experiments needed to achieve a certain detection limit was determined. Potential effects of EMF were investigated in long, medium and immediate reaction time for various field strengths up to 3000 V/m in the protein solution and different signals including GSM. Shifts of the thermal equilibrium corresponding to a temperature increase of only 0.1 K can be detected in minimum.

The results obtained so far suggest that the conformational equilibrium of GrpE is insensitive to weak electromagnetic fields. Future experiments will focus on the investigation of a potential influence of EMFs on protein dynamics by assessing shifts in the conformational equilibrium characteristics over an extended temperature range.

Introduction
Studies on the potential effects of electromagnetic fields (EMF) on different cell types have been examined alterations in complex cellular features and processes, such as genetic damage, proliferation, differentiation, apoptosis and gene expression. Mostly narrow-band signals such as emitted by mobile phones in the GSM or UMTS bands were applied. The biological analyses were carried out after the exposure to EMF. The results remain controversial, and in most cases their replication has proven difficult.

Previous studies on single proteins in simple protein-buffer systems have already reported effects of EMF on protein structure and folding kinetics. Since the molecular mechanisms are still unclear, a more mechanism-oriented approach is needed. In this study the isolated thermosensor protein GrpE of the Hsp70 chaperone system of Escherichia coli is exposed to EMFs while simultaneously monitoring its potential structural changes via circular dichroism spectroscopy. Thus, the point of observation becomes identical with the potential interaction site in space and time, a situation that will allow for the detection of even small effects of EMFs. By using isolated proteins, the complexity of the target is reduced from the cellular to the molecular level.

Heat-shock proteins (Hsp) are an important group of cellular stress response proteins. They act primarily as molecular chaperones to eliminate unfolded or misfolded proteins, which are generated under cellular stress. This stress response can be induced by various external factors, including increased temperature, chemicals, oxidative stress, heavy metals, ionizing and non-ionizing radiation and ultra-fine carbon black particles. An increased expression level of Hsp70 thus confers protection against cellular stress. Direct effects on the conformation of a component such as GrpE of the Hsp70 system of Escherichia coli (DnaK/DnaJ/GrpE) could therefore lead to a change in response to cellular stress.
Objective

GrpE undergoes a completely reversible conformational change upon an increase in temperature within a temperature range from 8 to 60 °C. Since the transition is fully reversible, a temperature-dependent conformational equilibrium ensues. Any direct effects on the conformation will result in a shift of this equilibrium.

In this study the hypothesis on potential direct effects from RF electromagnetic field exposure occurring in everyday situations on the structure of GrpE and its protein folding kinetics shall be investigated. The underlying questions are:

- Do electromagnetic fields shift the conformational equilibrium of GrpE?
- What is the observable effect size by the exposure setup used?

Furthermore, the threshold magnitude of EMF for a potential effect, as well as the frequency ranges where it occurs can be obtained. Provided that all thermal, electro-magnetical, chemical and optical conditions within irradiated volume are known and controlled this configuration should allow for discrimination of non-thermally induced structural changes from those caused by temperature changes. Once an effect is detected and its thresholds are found, the molecular mechanisms might be explored in detail by genetic engineering of GrpE, e.g. by deleting or introducing positively or negatively charged residues, mutational analysis of the hydrophobic core, etc.

Materials

**Circular Dichroism Spectroscopy**

A Circular Dichroism (CD) spectropolarimeter JASCO J-715 was used. CD measures the ellipticity of diluted protein solutions, i.e. the difference in absorption between left and right-handed polarised light. The CD signal of macromolecules provides information about their conformational equilibrium under certain environmental conditions (pH, temperature, etc.), i.e. the ratio of folded and unfolded proteins within the measurement volume. In particular the ellipticity signal is retrieved as an average from the total irradiated volume over a specified integration time. The different events expressed by instantaneous ellipticity are independent and superimposed by the noise of protein movement (Brownian motion) and the detector.

Circular dichroism (CD) spectroscopy is a well-established and widely-used experimental method to investigate structural properties of biomacromolecules. In particular, CD measurements provide information about the fractional population of different conformational states of macromolecules as function of the temperature [7]. It is known that CD radiation itself is not invasive, i.e. it does not alter the covalent and non-covalent structure of proteins and other bio macromolecules [3].

**Target protein GrpE**

The thermosensor protein GrpE of the Hsp70 chaperone system of *Escherichia coli* was chosen as the main target for the study since under defined conditions its conformational equilibrium uniquely depends on temperature and is reversible up to 60°C. The conformational transition corresponds to the melting of a long helix pair, which is readily observed by measuring far-UV circular dichroism. It depends on different factors...
such as the ionic strength of the buffer solution, the pH-value and solution viscosity. To ensure that the equilibrium is uniquely determined by temperature the pH-value of the buffer solution has to be kept neutral or slightly alkaline. Prior to the experiments a suitable potassium phosphate buffer concentration for providing a good environment for protein folding whilst minimizing the heat losses induced by the EMF are determined. GrpE was soluted in a 10 mM potassium phosphate buffer solution at a concentration of 10 μM.

Assuming that the conformational state of a protein changes within milliseconds, real-time measurements are possible. Within the specified temperature range the chemical and optical parameters are assumed to be homogeneous over the measurement volume and do not depend on temperature and time.

**Experimental unit**

An experimental unit consisting of a temperature-controlled exposure chamber directly installed in the measurement compartment of a spectropolarimeter was realized and tested (see Fig. 1). The exposure chamber was designed and characterized using numerical simulation together with experimental tools. The applied electric field strength is uniformly distributed over the illuminated volume with normalised standard deviation $\sigma < 0.1$ for all frequencies as recommended by [6].

The exposure chamber is fed using a signal generator together with an arbitrary waveform generator. The setup supports all commonly used study protocols and allows for arbitrary pulsed exposure durations with user-defined signals including DVBT, GSM and UMTS signals. A temperature probe is directly inserted into the cuvette containing the protein solution. The position of the probe is adjusted according to the centre of the optical beam measuring the CD-signal. A small sensor monitors the electric field strength. The temperature of the protein solution is controlled by computer-controlled thermostat circulating water bath. The signal generation units as well as the monitoring devices are all computer controlled.

The technical specifications for the exposure unit together with their uncertainty are summarized as follows:

**Figure 2:** SAR distribution within the solution on the left side together with the temperature distribution and induced convective flow. The distributions were calculated at 1.9 GHz and electric field strength of 3000 V/m.

**Figure 3:** On the left: numerical and experimental results for the EMF reflection coefficient for different cases; on the right: results of the difference in temperature at the measured location and the average one within the illuminated volume used for the correction at high electric field strength (1.9 GHz, 3000 V/m).
Frequency: ± 10.0 kHz (10 - 2000 MHz) 
Exposure Duration: ± 0.5 ms (1ms - limited by data storage size) 
Electric Field strength: ± 50 V/m (0 – 1200 V/m over whole frequency range – 3000 V/m max.) 
Volume: ± 10 µl (2 ml) 
Temperature: ± 0.1 K (5 – 95 °C) 
Optical wavelength: ± 1 nm (160 – 1200 nm) 

In general, a high reproducibility of all physical parameters was achieved. As an example the reproducibility of the conformational behaviour of the irradiated protein solution placed in the designed exposure chamber is shown in Fig. 3.

Numerical and experimental evaluation

A Comsol Multiphysics model including Maxwell, weakly compressed Navier-Stokes and Heat equation was utilised to compute the temperature distribution for different electric field strength excitation. In order to achieve controlled conditions the SAR distribution at different field strength and frequencies was calculated together with the induced temperature gradient and the induced convective flow within the solution. These results depicted in Figure 2 are used to define experimental protocols that for example are minimally influenced by temperature (< 0.1 K) and convective flow (< 0.1mm/s). In general electric field strength greater than 100 V/m will induce convective flow currents into the solution. An inhomogeneous temperature distribution within the CD illuminated volume is yielded resulting in a deviation between measured and average temperature of the volume. This flaw is corrected by the numerical model.

Measurements are conducted for EMF reflection coefficient for different loads and temperature behaviour at different location for several EMF excitations. Their results agree well with the numerical predictions as depicted in Fig. 3.
Methods

The overall uncertainty of the experimental system was evaluated. The data were tested for normal distribution using the Chi-square test and statistical analysis was performed with ANOVA method. The quantile plot on the right hand side shows the distribution of measurement values for different data pitch lengths (integration time of the spectropolarimeter) of 50 measurements each for a total integration time of 5 seconds. In Figure 4 the experimentally determined uncertainty for different integration times is summarized. Based on these results the number of experiments needed to achieve a certain detection limit was calculated and the results are shown in Figure 5. For example, more than 131 events are needed at a global temperature of 48 °C in order to detect a temperature change of 0.1 K at an integration time of 100 seconds with 95 % significance.

In order to assess the potential effects of EMF on the structure of proteins their CD signal have to be compared for the exposed and unexposed event provided that all physical conditions are constant at the illuminated volume including temperature distribution. Since the applied EMF will induce a temperature gradient in the solution two cases for comparison have to be considered:

1. constant temperature:
   This case is characterised by a low applied electric field strength resulting in a maximum temperature gradient smaller than 0.1 K. Repeated events of EMF exposed and unexposed experiments are compared directly by means of t-test. Any significant differences in the CD signal are consequently caused by a non-thermal EMF effects.

2. varying temperature:
   The EMF induced temperature rise has to be taken into account, thus the measured temperature has to be corrected. A series of EMF exposed events are discriminated by t-test to a predicted one derived from the unique temperature – CD signal relation of the proteins by means of statistical and numerical methods like Monte Carlo.

The target protein GrpE was tested for the several hypothesis and the protocols for the experiments carried out are summarized as follows:

<table>
<thead>
<tr>
<th>Hypothesis of</th>
<th>Cycle no. [1]</th>
<th>On cyc. [s]</th>
<th>Off cyc. [s]</th>
<th>Frequency [GHz]</th>
<th>$E_{\text{rms}}$ [V / m]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Irreversible effect of EMF</td>
<td>1</td>
<td>300</td>
<td>300</td>
<td>0.1, 1.0, 1.9</td>
<td>100, 160</td>
</tr>
<tr>
<td>2 Reversible immediate reaction on EMF</td>
<td>2000</td>
<td>0.1</td>
<td>2.9</td>
<td>1.0, 1.9</td>
<td>100</td>
</tr>
<tr>
<td>3 Reversible delayed reaction on EMF</td>
<td>various</td>
<td>various</td>
<td>various</td>
<td>0.1, 1.9</td>
<td>100, 2500</td>
</tr>
<tr>
<td>4 effect of EMF on folding dynamics</td>
<td>1</td>
<td>7200</td>
<td>7200</td>
<td>1.9</td>
<td>3000</td>
</tr>
<tr>
<td>5 Rev. del. reactions on GSM modulated EMF</td>
<td>30</td>
<td>5</td>
<td>25</td>
<td>GSM 900</td>
<td>201</td>
</tr>
</tbody>
</table>

Furthermore, the long-term stability of the protein solution has been assessed using 3' on / 3' off cycles each for 18 hours.

Results

In all experiments carried out until now no significant change in conformation of GrpE has been observed. No significant changes were observed also for the experiments using uplink GSM signals. The results of the data analysis for the experiments regarding hypothesis 1 - 4 are shown in Figure 6 and 7. Furthermore no changes in folding kinetics were observed as well. But the CD measurements of the protein solution containing GrpE show a remarkably high repeatability and a long-term stability up to 18 hours.
Figure 7: On the right: thermal equilibrium of GrpE is depicted for the unexposed and exposed case at 1.9 GHz, 3000 V/m with temperature correction; on the left: the CD signal of GrpE exposed to repeated on-off-sequences (3' on / 3' off) applying a field strength of 500 V/m at 1.9 GHz over 16 hours resulting in an EMF-induced temperature different between exposed and non-exposed of 2 K.

Conclusion

The designed exposure chamber allows for controlled and reproducible EMF exposure of proteins in diluted solution at specified temperature and electromagnetic field strength whilst monitoring its CD signal in real time. The number of repetitions of exposure events necessary to identify significantly an effect with a predetermined detection limit was assessed. Thus, conditions and uncertainty are known in order to detect potential effects in long or immediate reaction time. Shifts of the thermal equilibrium corresponding to a temperature increase of only 0.1 K can be detected in minimum.

The target GrpE protein exhibits long term stability and is highly sensitive to temperature changes within the physiological range of 8 - 60 °C. The results obtained so far suggest that the conformational equilibrium of GrpE is insensitive to electromagnetic fields. Future experiments will focus on the investigation of a potential influence of EMFs on protein relaxation / adaption time on temperature differences.

References


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