

Non-Thermal Effects in the Microwave Induced Unfolding of Proteins Observed by Chaperone Binding

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We study the effect of microwaves at 2,450 MHz on protein unfolding using surface plasmon resonance sensing. Our experimental method makes use of the fact that unfolding proteins tend to bind to chaperones on their unfolding pathway and this attachment is readily monitored by surface plasmon resonance. We use the protein citrate synthase (CS) for this study as it shows strong binding to the chaperone alpha crystallin when stressed by exposure to excess temperature. The results of microwave heating are compared with the effect of ambient heating and a combination of ambient and microwave heating to the same final temperature. We study the temperature distributions during the heating process. We show that microwaves cause a significantly higher degree of unfolding than conventional thermal stress for protein solutions heated to the same maximum temperature. *Bioelectromagnetics* 29:324–330, 2008. © 2008 Wiley-Liss, Inc.

Key words: citrate synthase; alpha crystallin; surface plasmon resonance; high frequency electromagnetic field; non thermal effects

INTRODUCTION

Microwaves from devices such as mobile phone transmitters/receivers, satellite transmitters, radar, radio/TV transmitters, video display terminals and microwave ovens are ubiquitous in modern life. Proteins are the mediators of cell function and studies of the effect of microwaves on them are therefore of significance in managing the population's exposure to environmental electromagnetic radiation. A heat stress delivered by microwaves to an organism necessarily involves exposure of its proteins to oscillating electric and magnetic fields, raising the possibility that non-thermal, that is direct field mediated, effects could result in different outcomes for the proteins than would arise from a heat stress delivered by heat conducted from the environment.

A related question in organic chemistry has been addressed in a recent review by de la Hoz et al. [2005], who examined the evidence for non-thermal effects of microwaves on chemical reactions. There are many observations of the acceleration of chemical reactions by exposure to microwaves and, in some cases, the effect is dramatic. Most of the effects are explained by greater than expected temperature rises, including "hot spots" caused by strong absorption of microwave energy selectively by parts of the system. A number of cases have been identified, however, where the outcome of a chemical reaction is qualitatively different in the presence of a microwave field providing strong evidence that, at least in some

cases, the oscillating electric and magnetic fields can change the course of the reaction. This could occur, for example, by an interaction between the microwave electromagnetic fields with polar intermediates. If the population of these intermediates is changed by this interaction, the contribution to the reaction from pathways that involve these intermediates would be altered.

Evidence has been presented [Bohr and Bohr, 2000a,b; Coptly et al., 2005] that the effect of microwaves on protein conformation and function is not the same as the effects of ambient heating with conducted heat, at the same temperature. In the work of Bohr and Bohr, the effect of microwaves on a cold denaturing protein was studied. It was found that microwaves accelerated cold denaturing when the temperature

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was decreasing but accelerated refolding when the temperature was increasing. This is contrary to the effects of heating in thermodynamic equilibrium which would promote refolding in both cases. In the work of Coptý et al. [2005] it was found that the effects of microwaves on the fluorescence of a green fluorescent protein was greater than could be explained on the basis of the temperature changes alone.

In a study by de Pomerai et al. [2003] it was found that exposure to microwave radiation of an average SAR of 15–20 mW/Kg enhances the aggregation of bovine serum albumin *in vitro*. Microwave exposure was also reported to promote amyloid fibril formation by bovine insulin at 60 °C. These results imply that protein conformation is affected by microwave exposure, but the temperature changes that accompany the exposure are not sufficient by themselves to cause the effects observed. More recently, Wang et al. [2006] found that microwave radiation exposure slightly increased the expression of the heat shock protein HSP70 and significantly increased phosphorylation of the heat shock protein HSP27. The phosphorylation effects were not consistent with the effects of the temperature rise alone.

In this article, we present the results of an experimental investigation of microwave induced unfolding of the protein citrate synthase as measured by its binding to a chaperone. The protein was exposed to microwaves only, conducted heat only and combinations of conducted heat and microwaves. Control samples without exposure were made for each exposed sample. Surface plasmon resonance was used to detect attachment to the chaperone, alpha-crystallin, which was immobilized on a Biacore chip. Additional control experiments were conducted in which there was no chaperone on the binding surface.

MATERIALS AND METHODS

Materials

Bovine α -crystallin and porcine heart CS were obtained from Sigma–Aldrich. One milligram per milliliter bovine α -crystallin in HBS buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20; Biacore) was diluted in 10 mM NaCH₃COOH pH 4.5 to a final concentration of 200 μ g/ml and immobilized on a CM5 Biacore sensor chip (Biacore AB, Uppsala, Sweden) via amine coupling.

Methods

Exposure of the protein. The citrate synthase solution was placed in an Eppendorf tube of volume 80 μ l for the

exposures. A microwave oven cavity, operating at 2.45 GHz, was used for the microwave exposures. The exposures were delivered in the form of a series of 50 Hz pulses of microwave power administered over a time between 10 and 20 s.

A shelf was designed as shown in Figure 1 from high density polyethylene with holes sized to fit the Eppendorf tubes. The holes were scanned by measuring the average temperature rise of the same water filled Eppendorf tube at each location. The hole having the maximum temperature rise was selected for the experiment. A water ballast was placed under the shelf in a fixed spot as shown in the figure. The water ballast stabilized the field in the microwave cavity. The temperature rise in the Eppendorf tube was measured using a Luxtron fluoroptic probe STF2 (Luxtron Corporation, model FOT Lab Kit Serial 61312), a device which has a temperature sensing fiber optic of small thermal mass. This type of probe does not perturb the microwave field as it is constructed of silica which does not absorb microwave power at the frequency

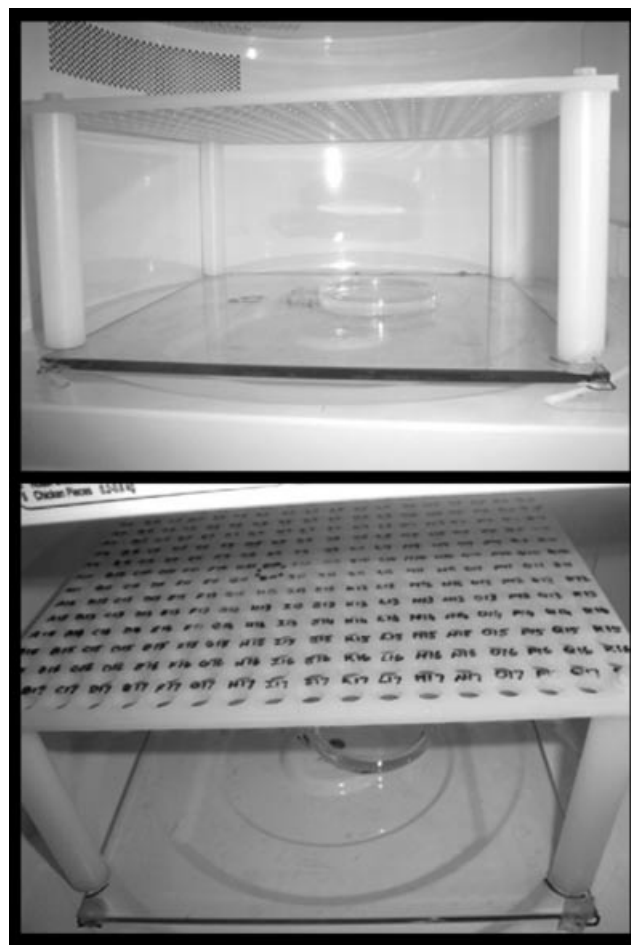


Fig. 1. The HDPE rack inside the microwave with the water ballast underneath.

used. For all exposures, the Luxtron probe was used to sense the temperature of the protein solution near the end-tip of an Eppendorf tube. The Luxtron probe senses the temperature through a fluorescent phosphor of dimension 0.8 mm placed at the tip of the probe. Since the silica fiber has a low thermal conductivity of the same order as the solution, the presence of the fiber will cause minimal perturbation to the temperature distribution. The probe therefore reads average temperature over a volume of side dimension 0.8 mm at the location of the sensing phosphor. Temperatures were chosen to cover the same range as the microwave exposures. The distribution of temperature rise throughout the solution in the Eppendorf tube may be different, as discussed in the results. After exposure, the protein solution was immediately passed through the flow cell of the Biacore. For exposure to temperatures above 43 °C by ambient heating, the Eppendorf tube was placed in a water bath at the desired temperature.

In the following set of experiments, CS was prepared at concentrations of 100 and 150 µg/ml and its binding to α -crystallin was studied after subjecting CS to (a) microwave only, (b) water bath only and (c) a combination of water bath and microwave heating.

- (a) *Microwave exposure*: CS was heated in a microwave oven for durations of 10, 15, and 20 s. Fifty microliters of the exposed CS were then run over a control flow cell and a flow cell containing immobilized α -crystallin at a flow rate of 30 µl/min. The experiments were run with the Biacore system at a temperature of 25 °C. The average specific absorption rate (SAR) is 4.85 kW/kg. The magnetron source of the transformer type microwave oven produces a sharply peaked waveform with the frequency of the mains supply voltage (50 Hz) [Matsumoto et al., 2005]. The waveform produced by the oven was examined using a dipole antenna coupled to an oscilloscope. The peak to average field amplitude ratio was in the range between 3:1 and 8:1.
- (b) *Water bath heating*: The water bath temperature was set to a preset temperature in the range of 34 to 82.5 °C. CS was then allowed to remain at the preset temperature for 5 min and was then flowed through the Biacore flow cell at a flow rate of 30 µl/min. The volume injected was 50 µl. The Biacore temperature was 25 °C as in the case of microwave heating.
- (c) *Water bath heating followed by microwave exposure*: CS was heated in a water bath for 5 min to preset temperatures in the range of 29.5 to 44 °C, directly followed by a microwave exposure for periods of 10, 15, and 20 s.

Measurement of Binding to Immobilized Chaperone

The α -crystallin was immobilized on the surface of a BiacoreTM surface plasmon resonance chip, type CM5, to give a convenient readout of the degree of binding. The resonance unit (RU) is a convenient arbitrary unit related to the angle shift in the minimum in reflectance due to the surface Plasmon resonance.

The Biacore CM5 chip allows four separate flow cells to be operated. Amine coupling of the chaperone to the dextran coated sensor chip was achieved by activating the surface of the chips with equal volumes of 50 mM N-hydroxysuccinimide (NHS)/200 mM N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide (EDC) (Biacore, AB, Uppsala, Sweden). The α -crystallin solution (200 µg/ml) was allowed to flow over the chip for 6 min at a rate of 5 µl/min. The surface was blocked by ethanol amine. α -crystallin was immobilized on the sensor chip to give an attachment signal increase of approximately 5000 RU. A control surface without α -crystallin was prepared by activating and blocking without running the α -crystallin solution.

The standard operating procedure for each experiment consisted of an initial flow of HBS buffer over the Biacore chip, followed by the injection and flow of protein containing solution (association phase) and finally a flow of fresh HBS buffer again (dissociation phase). The change in RU during each of these flow stages was used to quantify the attachment of CS to α -crystallin (during the association phase) and to examine the amount of residual binding (during the dissociation phase). For every experiment using a flow cell containing immobilized α -crystallin on the chip surface, another (control) experiment was carried out using the same procedures except that the step for immobilization of α -crystallin was omitted.

CS was allowed to flow over one flow cell containing immobilized α -crystallin and over a control flow cell. The flow rate was 30 µl/min and the injected volume was 50 µl in both cases. The Biacore internal temperature was set at 25 °C.

RESULTS

Temperature Distributions in the Samples

Figure 2 shows a typical temperature profile of the solution as a function of time during a microwave exposure compared with a typical temperature profile for a solution heated by conduction by immersion in a water bath, measured near the bottom of an Eppendorf tube. Differences in the temperature-time profiles are a higher maximum heating rate in the microwave case,

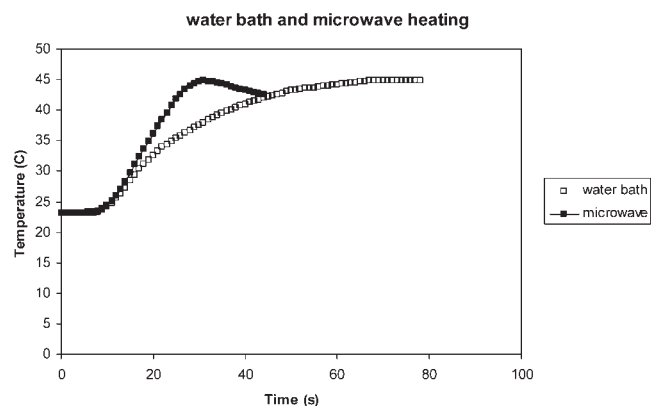


Fig. 2. The temperature profile of a protein solution during microwave exposure for 20 s (solid squares) and the temperature profile of the same protein solution heated in a water bath for 5 min (open squares). Both profiles reach a temperature of 44.8 °C.

typically 0.7 °C/s, compared to 0.33 °C/s for the conducted case.

In order for comparisons to be made, the distribution of temperature within the Eppendorf tube must be known, to ensure that a measure of the final temperature near the bottom of the Eppendorf tube does not give any undesirable bias with respect to the average temperature in the cell.

Binding of Protein to the Chaperone

The results for the binding of the exposed protein to α -crystallin are shown in Figure 3. The plot shows the difference in binding between the exposed and control samples as a function of the final temperature, measured at the end of the exposure and prior to flowing through the Biacore cell. The protein heated by contact with the

water bath shows binding that increases slowly with the temperature up to approximately 63 °C, after which many samples show a strong increase in binding. In another experiment (data not shown), the temperature of 63 °C was identified as a temperature at which there was an onset of strong binding to the chaperone, believed to correspond to the formation of a monomeric unfolding intermediate. The corresponding plot for the microwave exposed samples shows that the binding is generally higher at the same final temperature than for the water bath heated samples and does not show the clear threshold effect for temperatures above 63 °C. The fact that the strong binding is occurring after exposure to a lower final temperature suggests that microwaves cause a distinctly different response than water bath (ambient) heating. The absence of the threshold unfolding temperature for microwave exposure indicates that the unfolding intermediate that gives rise to the strong binding is in fact entered at a lower temperature than for ambient heating.

As a simple means of confirming that the microwave response is different from the response to ambient heat, we used the fact that the maximum temperatures reached in both the ambient heating and the microwave heating uniformly cover the temperature band between 43 and 82 °C. The mean of the final temperatures for microwave heating was 57.4 °C and the mean of the final temperatures for ambient heating was 61.7 °C. If we regard each set of results as a sample of a population in which the temperature randomly covers the range 43–82 °C, we can test the hypothesis that the microwave heated results and the ambient heated results come from the same population. To do this we calculate the statistic “*t*.”

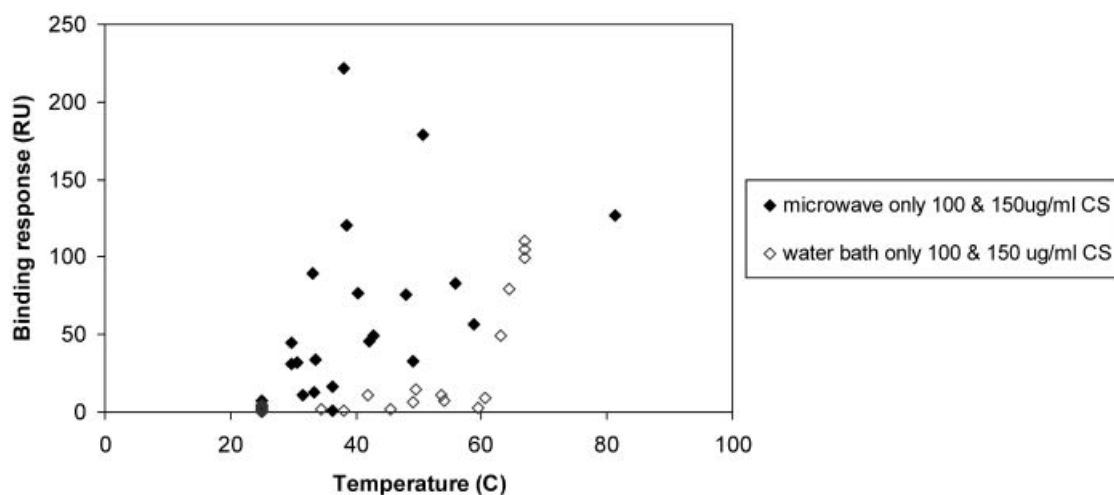


Fig. 3. The difference in binding of citrate synthase (CS) to α -crystallin minus binding to a control surface versus final temperature. The open diamonds show the binding for CS samples heated in a water bath for 5 min. The solid diamonds show the binding for CS samples heated using microwaves.

Table 1 gives the observations and the result for t in this comparison. With a high degree of confidence ($>99.9\%$) the two samples (microwave and water bath heating) cannot be considered as coming from the same population, confirming that there is a substantially different behavior after exposure to microwaves.

TABLE 1. The Exposure Times of the System, the Final Temperature and the Binding for Microwave Heated Samples (a) and Ambient Heated Samples (b) and a Combination of Microwave and Ambient Heated Samples (c)

	Temp (°C)	binding
a: Microwave duration		
20	45.03	57
20	47.73	106
20	49.13	36
10	50.62	205
20	55.82	123.1
15	58.7	37.3
20	70.95	61.6
20	81.41	89.6
b: Water bath duration		
300	45.5	14.4
300	46.5	16.2
300	49	21.4
300	49.5	27
300	51.5	16.6
300	54	13.5
300	58	12.3
300	59.5	17.3
300	60.5	18.9
300	63	56
300	64.5	86.2
300	67	112.8
300	67	99.4
300	67	94.4
300	72	18.9
300	74.5	18
300	80.5	27.5
300	82.5	20.3
c: Microwave part of H + M duration		
20	44.26	67
10	45.07	34.7
10	45.2	49.7
10	45.23	49
20	45.44	56.4
10	49.65	24
20	50.74	17
10	50.94	175
10	50.94	175
20	51.46	37.8
10	53.47	69
20	54.9	238
20	65.84	81.4
20	66.25	294
20	70.95	61.6

$t = 2.99$ in favor of microwave heating over water bath heating.
 $t = 0.18$ in favor of a combination of water bath heating + microwave heating over microwave heating.
 $t = 2.71$ in favor of a combination of water bath + microwave heating over water bath heating.

The same test can be used to compare the samples that have been subjected to the combination of microwave and ambient heating with those subjected to ambient heating only. The result for t , shown in Table 1, indicates that the hypothesis that the two samples are from the same population is even more strongly denied. However, when we compare the results for microwave only heating with the results for the combination of microwave and ambient heating, we cannot deny the hypothesis at less than the 50% confidence level.

DISCUSSION

The spatial distribution of absorbed power in an electrolyte solution is usually not uniform for a conductive sample in a microwave or radiofrequency field. It is necessary to assess the possibility of “hot spots” in our sample volume where the local SAR values might be significantly elevated above the values at the detection point. At low frequencies, in the range of 57 MHz Yang et al. [1984] found that the distribution of SAR in spherical distributions of tissue was maximum near the periphery of the sphere for high conductivities, and for small spheres with low conductivity the SAR was uniform throughout the sphere. Burkhardt et al. [1996] found that for a cylindrical sample with heights of 2.4 mm and diameters of 52 mm and with conductivity 1.8 S/m that the absorbed power at 835 MHz was non-uniform, with most power absorbed at the surfaces of the sample. Weil [1975] at frequencies ranging from 0.1 to 10 GHz found that for concentric spheres with conductivities approximating human head tissues, ranging from low conductivity at the outer regions (bone) to high conductivity, over 1 S/m in the inner regions, that the absorbed power was increasingly maximized at the center of the sphere as the sphere diameter decreased. In our case, where the vacuum wavelength is 12.25 cm, significantly larger than the sample, the distribution of absorbed power is expected to be approximately uniform for our electrolyte conductivities which are of the order of 1 S/m. To confirm this expectation, we measured the distribution of temperature rises for our samples that actually occurred during exposure to microwaves. The temperature rise in the Eppendorf tube resulting from microwave exposure was examined with a Luxtron immersion probe at three different heights. The results for the temperature rises are shown in Table 2. There is a slight downward gradient in the temperature rise with distance from the bottom of the tube. These results show that there was no “hot spot” in the tube and a measurement near the bottom of the tube can be taken as the upper limit for the temperature rise. In

TABLE 2. Table Showing the Experimental Measured Temperatures at Three Different Depths of the Protein Solution in an Eppendorf Tube

	Bottom of the solution	Middle of the solution	Top of the solution
Average temperature rise (°C)	1.85 ± 0.4	1.38 ± 0.8	1.30 ± 0.49
Distance from top of the solution (cm)	0.4	0.2	0.06

the exposure of our samples for the protein studies the temperature probe was located near the bottom of the sample. Our reported temperatures are therefore the highest temperatures for any point in the sample.

Water Bath Heated Samples

A small volume of 80 µl of solution was heated in the same Eppendorf tube as for the microwave exposures. The solution of the heat diffusion equation [Carslaw and Jaeger, 1947] shows that the temperature at the center of a sphere heated by conduction from its outer surface held at constant temperature follows an exponential trend with time and asymptotes to the outside temperature. The temperature-time plot of Figure 2 shows that the solution temperature has reached the outside temperature comfortably within the exposure time. This is indicative that a uniform distribution of temperature throughout the whole sphere has been reached. The temperature at the center of the Eppendorf tube was measured to confirm this. The use of the water bath temperature as the final temperature is therefore justified.

The profiles for ambient heating and microwave exposure are different in detail (see Fig. 2) so it is important to determine whether this difference is a confounding factor in the statistical analysis above. To confirm that there is no effect which could favor unfolding in the microwave exposure, we now calculate the total degree of unfolding that would be expected on the basis that it is driven by temperature alone. The unfolding rate k of pig heart CS has been measured as a function of temperature [Gerike et al., 1997] and has been found to satisfy the Arrhenius relation for a simple two state reaction:

$$k = Ae^{-E_a/RT},$$

where A is the pre-exponential factor or frequency factor, E_a is the activation energy, T is the temperature in Kelvin and R is the gas constant.

The amount of unfolded protein can be calculated by integrating the rate constant for both temperature profiles of Figure 2. The calculated ratio of the amount of unfolded CS for water bath heating to the amount of unfolded CS for microwave heating was found to be 2.6.

This is in fact an underestimate because the duration of water bath heating in all cases was longer than the 80 s time period shown in Figure 2.

The amount of unfolded protein for the water bath heated sample should therefore be higher than that of the microwave heated samples even though the maximum temperature reached is the same. However, our experimental results show that the microwave heated samples have higher binding to the chaperone α -crystallin than the water bath heated samples when compared at the same maximum exposure temperature, an observation that cannot be explained by the difference in the temperature profiles.

A possible explanation for the enhanced unfolding that occurs during a microwave exposure is that energy is delivered selectively by resonant absorption of electromagnetic energy to some vibrational states of the protein or its bound water envelope, driving these states out of thermal equilibrium with the remainder of the vibrational states. The direct action of an oscillating electric field on a protein in solution has been simulated by Budi et al. [2005] using force field molecular dynamics. The electric field can cause unwinding of peripheral regions of the protein backbone and thereby promote unfolding. Another avenue for site specific absorption is through water molecules bound to the protein. Bound water absorbs in the range of 10 MHz to 10 GHz [Hill et al., 1978] and comprises the surface water and the water of hydration. Water of hydration starts absorbing at 50 MHz and reaches saturation at 2 GHz [Grant et al., 1975]. The pulsed nature of the microwave field used in this study increases the potential for the direct action of the electric field. The peak value of the electric field is many times higher than the peak field that would apply to a continuous microwave exposure of the same average absorbed power.

CONCLUSION

This work compares the effects of microwave exposure, ambient heating and a combination of both on citrate synthase unfolding by measuring its binding to the chaperone α -crystallin. The results show with a high level of confidence that unfolding occurs at significantly lower temperatures whenever microwave heating is

used. This supports the hypothesis that microwaves have a non-thermal effect on protein conformation that could take the form of a direct interaction of the electromagnetic field with the protein or its water of hydration.

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