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Non-thermal effects of microwaves on proteins: thermophilic enzymes as model system

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Abstract Two thermophilic and thermostable enzymes, isolated from *Sulfolobus solfataricus*, *S*-adenosylhomocysteine hydrolase and 5′-methylthioadenosine phosphorylase, were exposed to 10.4 GHz microwave radiation in order to discriminate between thermal and non-thermal microwave effects. The exposure causes a non-thermal, irreversible and time-dependent inactivation of both enzymes; the inactivation rate is related to the energy absorbed and is independent of the enzyme concentration. The influence of salts on enzyme inactivation has also been investigated. Conformational changes of *S*-adenosylhomocysteine hydrolase, detected by fluorescence and circular dichroism techniques, suggest that microwaves induce protein structural rearrangements not related to temperature.

Key words: Thermophilic enzyme; Microwave radiation; Non-thermal effect; *S*-Adenosylhomocysteine hydrolase; 5′-Methylthioadenosine phosphorylase

1. Introduction

During the last few decades, the use of microwave radiation has greatly increased in radar and communication systems as well as in food-processing technology and in other industrial applications. The development of consumer and medical microwave devices for clinical diagnosis and therapy also has prompted widespread interest and has stimulated much research on the mechanisms of interaction of microwave radiation with living organisms [1–5]. According to the literature, two types of effects can be ascribed to microwaves, i.e. thermal and non-thermal [1,2,4,5]. The thermal effects are related to the heat generated by the absorption of microwave energy by the water medium or by organic complex systems, both characterized by a permanent or induced polarization. At present, very little is known about the molecular mechanisms involved in the putative non-thermal effects which could involve direct energy transfer from the electromagnetic field to the vibrational modes of macromolecules [6] altering their conformation.

Many non-thermal effects following exposure of biosystems to microwaves have been reported in recent years; among them changes of the activity of Ca^{2+}-dependent K^+ channels [7], alterations of membrane structure and function [8,9], permeability modifications of liposomes [10,11] and isolated cells [12] have been described. On the other hand, some authors have questioned the very existence of non-thermal microwave effects [4,13,14].

In particular the results obtained on enzyme systems are so far controversial, probably due to the experimental difficulties in the proper control and monitoring of the temperature. No measurable non-thermal effect on catalytic activity has been observed in a number of isolated enzymes irradiated in vitro [15–18]. Conversely, other enzyme systems such as lymphocyte protein kinases [19], hepatoma cell ornithine decarboxylase [20] and acid phosphatase [21] respond to low or high intensity and amplitude-modulated microwave fields. Moreover, a significant inhibition of red cell Na^+/K^+ ATPase, presumably related to conformational changes of the protein, has been reported [22].

In the present work a novel experimental approach aimed to discriminate between thermal and non-thermal effects is described using purified thermophilic enzymes as model system. The thermophilicity and thermostability of such molecules allow high intensity microwave exposure with minor temperature interference on the enzyme stability, thus permitting the use of proper controls at high temperatures.

This paper reports the effects of 10.4 GHz microwave exposure on the stability of two thermophilic enzymes involved in polyamine and *S*-adenosylmethionine metabolism [23], i.e. *S*-adenosylhomocysteine (AdoHcy) hydrolase and 5′-methylthioadenosine (MTA) phosphorylase, purified from *Sulfolobus solfataricus* [24,25], a thermophilic microorganism belonging to the Archaea [26]. Furthermore, data on the influence of microwaves on the conformation of AdoHcy hydrolase are reported.

2. Materials and methods

2.1. Microwave exposure

The exposures were carried out in a waveguide system where accurate microwave dosimetry and thermal control were provided. The microwave circuit has been described in detail [27]. Incident, reflected and transmitted powers (P_i, P_r and P_t respectively) were continuously monitored during the experiments. The absorbed power (P_a) was evaluated as the difference between the incident power and the sum of reflected and transmitted powers [$P_a = P_i - (P_r + P_t)$]. The specific absorption rate (SAR) was calculated as the ratio between the absorbed microwave power (W) and the mass (g) of the sample, and various SAR levels were used. Preliminary tests showed a good matching (low P_r) between feeding circuit and sample holder at 10.4 GHz; therefore, all the experiments were carried out at this frequency, and no frequency-dependent effect was investigated. A cone-tipped plastic test tube, containing the enzyme solution, was inserted axially into the sample holder waveguide that was surrounded by a water jacket at 30°C in order to give a thermally stable environment. During the

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Abbreviations: AdoHcy, *S*-adenosylhomocysteine; MTA, 5′-methylthioadenosine; SAR, specific absorption rate

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exposure, the temperature was continuously monitored by means of two thermocouples (Physitemp, PT-6) and a digital precision thermometer (Bayley TH6D). The temperature probes being perpendicular to the electric field, the temperature measurement was not affected by the microwave radiation. For the same reason, a negligible electromagnetic field distortion could be assumed. Any change of the sample temperature value, owing to small physical changes occurring in the sample holder section, was compensated by varying the incident power. In addition, in order to avoid thermal disuniformities in the sample solution, a motorized glass stirrer was used. In these conditions, the maximum temperature difference within the sample was no more than 0.5°C. The designed temperature was reached within 2 min in both the sample and the control. The heating kinetics of the samples, measured by the thermocouples, were comparable with those of controls.

2.2. Enzyme assays and protein determination

AdoHcy hydrolase activity was assayed following the synthesis of [8-^{14}C]AdoHcy from [8-^{14}C]adenosine in the presence of homocysteine. The assay was performed as described by Porcelli et al. [24].MTA phosphorylase activity was determined by measuring the formation of [methyl-^{14}C]5-methylthioribose-1-phosphate from [methyl-^{14}C]MTA. The assay procedure has been described [25].

Protein concentration was estimated according to Bradford [28] using human γ-globulin as standard.

2.3. Microwave irradiation

Unless otherwise stated, 300 μl solution (0.3 mg/ml) of AdoHcy hydrolase or MTA phosphorylase in 10 mM Tris-HCl buffer, pH 7.4, was exposed to 10.4 GHz microwave radiation at different temperatures in the 70–90°C range. In these conditions the SAR varied in the 1.5–3.1 W/g range. At different time intervals, 50 μl enzyme samples were withdrawn from the exposure cell and assayed. The residual enzymatic activity was then calculated as a percentage of a control incubated at the same temperature in a water bath.

2.4. Spectral measurements

Fluorescence measurements were carried out on a Perkin-Elmer MPF-66B spectrofluorometer in the range of fluorescence linearity. The absorbance of all solutions was 0.02–0.15 at the excitation wavelength.

Circular dichroism (CD) measurements were carried out on a Jobin Yvon Mark III spectropolarimeter. The absorbance of protein samples used for CD measurements was about 0.125 at 280 nm. The CD spectra were analyzed in the 200–250-nm region.

3. Results and discussion

3.1. Effects of microwave radiation on the enzyme stability

AdoHcy hydrolase and MTA phosphorylase from *S. solfataricus* have been purified [24,25], extensively characterized [24,25], and cloned [29,30] in our laboratory. Both enzymes are endowed with high thermophilicity and thermostability, as well as with a remarkable resistance to organic solvents, protein denaturants, and detergents, even at elevated temperatures [24,25].

AdoHcy hydrolase and MTA phosphorylase were exposed to 10.4 GHz microwave radiation of elevated intensity (SAR, 1.5–3.1 W/g) in a temperature range from 70°C to 90°C. As reported in Fig. 1a,b, irradiation causes a loss of enzymatic activity in both enzymes as a function of the exposure time at the experimental temperatures utilized. The extent of inactivation is different for the two enzymes. In the temperature range studied, AdoHcy hydrolase appears more sensitive than the more thermostable MTA phosphorylase. In fact, at 90°C AdoHcy hydrolase retains only 18% activity after 40 min irradiation compared to a control incubated at the same temperature without irradiation. In the same conditions MTA phosphorylase still retains 78% activity after 40 min and reaches greater inactivation (58%) only after 90 min.

The enzymatic inactivation observed should be ascribed to a non-thermal microwave effect since AdoHcy hydrolase appears fully active after 90 min of incubation at 70°C and after 30 min at 90°C [24] and MTA phosphorylase is completely stable for up to 2 h incubation at 100°C [25].

Since the water jacket surrounding the sample holder waveguide was maintained at a constant temperature, the attainment of the experimental temperatures was brought about by the different levels of energy absorbed by the samples. As shown in Fig. 1a,b, the enzyme activity decreases with the increase in microwave power absorbed per unit mass (SAR). Further comparison of the two figures points out that the effect exerted by the microwaves depends on the structure of

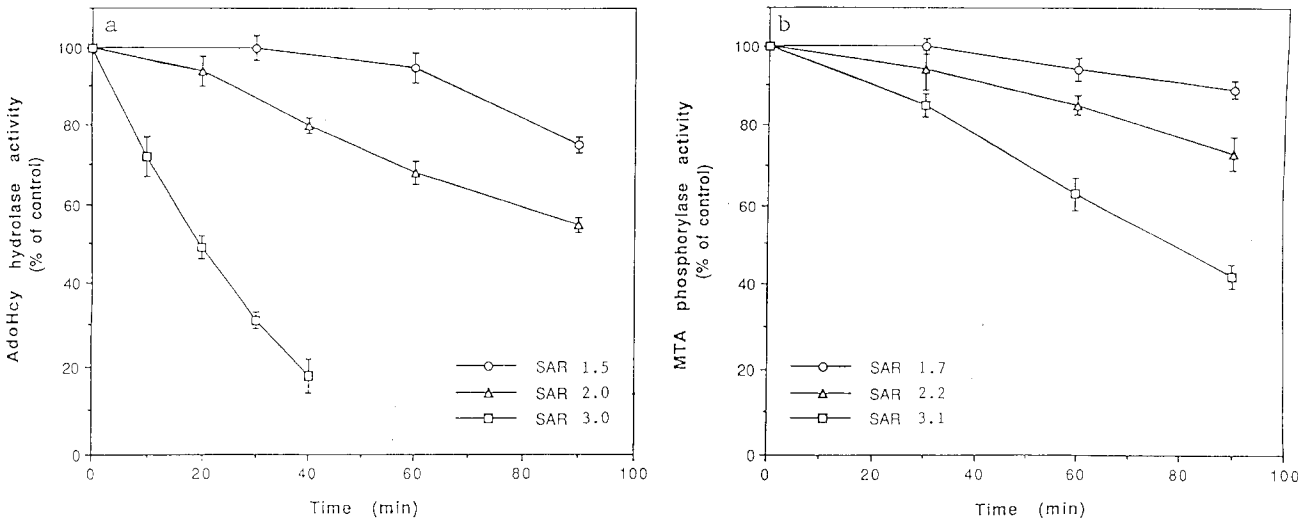

Fig. 1. AdoHcy hydrolase (a) and MTA phosphorylase (b) activity after exposure to 10.4 GHz microwave radiation. SAR was calculated as the ratio between the absorbed microwave power (W) and the mass of the sample (g). a: AdoHcy hydrolase activity as a function of the time of exposure; b: MTA phosphorylase activity as a function of the time of exposure. The enzymes were exposed to microwaves at 70°C (○), 80°C (△), and 90°C (□). At different times of exposure, aliquots were withdrawn and assayed for enzyme activity and compared to controls incubated at the same temperature without irradiation. All experiments were carried out in triplicate, and both the average values and the error bars are given in the figure.

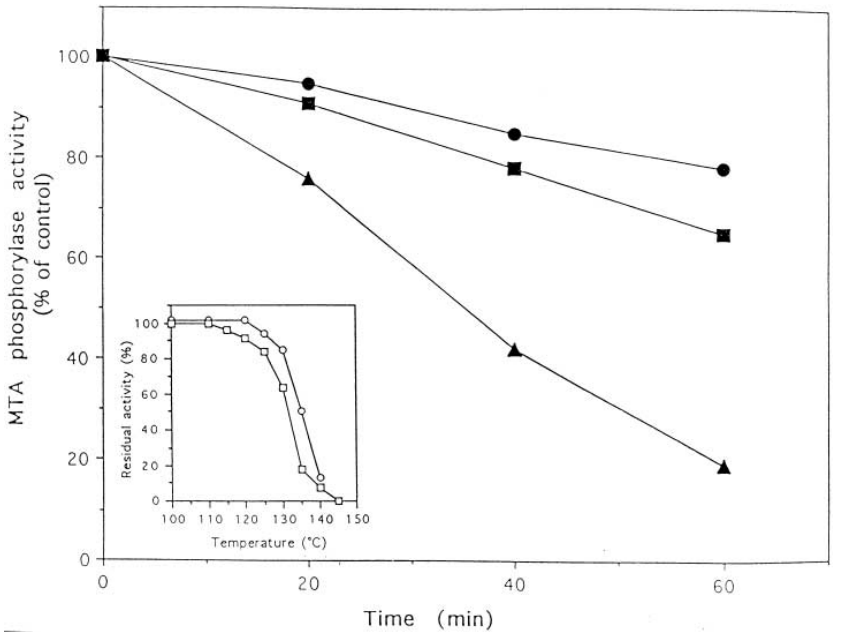

Fig. 2. Effects of salts on MTA phosphorylase inactivation. Microwave-exposed MTA phosphorylase activity (■); microwave-exposed MTA phosphorylase activity in the presence of 250 mM KCl (▲), and in the presence of 250 mM KH_2PO_4 (●). At different times of exposure at 90°C (SAR, 3 W/g), aliquots were withdrawn and assyed for enzyme activity. In the inset is reported the residual MTA phosphorylase activity after 10 min of preincubation in the presence (○) and absence (□) of 250 mM KH_2PO_4, as a function of temperature. Enzyme thermostability was tested by incubating the protein in sealed glass vials at temperatures between 100 and 145°C. Activity was assayed in standard conditions by the addition of 50 μl aliquots from each preincubated sample.

the specific protein; in fact, at 90°C, when the power absorbed (SAR) by the two enzymes is similar, the decrease of the activity values is quite different.

The effect of the electromagnetic field does not depend on the enzymatic concentration of the sample, since experiments carried out at different protein concentrations, ranging from 0.01 to 0.3 mg/ml, did not reveal any change in the inactivation kinetics (data not shown).

The influence of salts on the enzyme inactivation was studied by subjecting AdoHcy hydrolase and MTA phosphorylase to 10.4 GHz microwave irradiation at 90°C (SAR, 3 W/g) in the presence of 250 mM KCl or 250 mM KH_2PO_4.

The two thermophilic enzymes show different behavior; the addition of KCl or KH_2PO_4 to the enzymatic solution exposed to microwaves does not cause any further effect on AdoHcy hydrolase inactivation (data not shown). On the other hand, KH_2PO_4 exerts a moderate protection towards microwave inactivation of MTA phosphorylase while KCl enhances the inactivation process (Fig. 2). Furthermore, a similar experiment, performed with 250 mM NaCl and 250 mM Na_2SO_4 indicates that after 1 h irradiation of MTA phosphorylase at 90°C (data not shown), Na_2SO_4 exerts a moderate protection (76% residual activity) while NaCl causes an increase of the inactivation of the enzyme (34% residual activity).

The mechanism by which KCl or NaCl increases the susceptibility of MTA phosphorylase to microwave irradiation is at present difficult to interpret and deserves further investigation. Conversely, the protection against microwave inactivation exerted by phosphate or its analog sulfate could be ascribed to their role as substrate of MTA phosphorylase. It is known that the binding of substrates results in the protection of the enzymes against the inactivation caused by physical or chemical agents such as temperature or proteolytic enzymes [31]. Therefore, in order to evaluate the possible protective effect of phosphate on the thermostability of MTA phosphorylase, we carried out short-term kinetics of thermal denaturation of the enzyme in the presence and in the absence of 250 mM KH_2PO_4. As shown in the inset in Fig. 2, from the diagram of the residual activity after 10 min of preincubation as a function of temperature it is possible to calculate a transition temperature (apparent Tm) of 132°C. This value increases to 135°C when the enzyme is preincubated with 250 mM KH_2PO_4, thereby indicating a significant protection by the phosphate.

On the basis of the reported results, it is possible to hypothesize that the binding of the substrate increases the conformational stability of the enzyme thus modifying its susceptibility to microwave radiations.

3.2. Microwave effects on AdoHcy hydrolase structure

The observed inactivation of AdoHcy hydrolase and MTA phosphorylase by microwave exposure suggests that the structure of both enzymes was directly affected by the electromagnetic field.

Fluorescence and circular dichroism have been used to investigate the possible conformational changes induced by microwaves on AdoHcy hydrolase. Fig. 3 shows the fluorescence emission spectra of irradiated AdoHcy hydrolase when excited at 280 nm and 340 nm, in comparison with the emission spectra of the respective controls. The fluorescence emission in the range 300–400 nm upon excitation at 280 nm mainly arises from the protein intrinsic fluorophores, i.e. tryptophan and tyrosine residues. Instead, the fluorescence emission, when exciting at 340 nm, is related to the enzyme-bound NADH. *S. solfataricus* AdoHcy hydrolase, in fact, is composed of four identical subunits and contains four molecules of tightly bound NAD per tetramer, of which about 40% is in

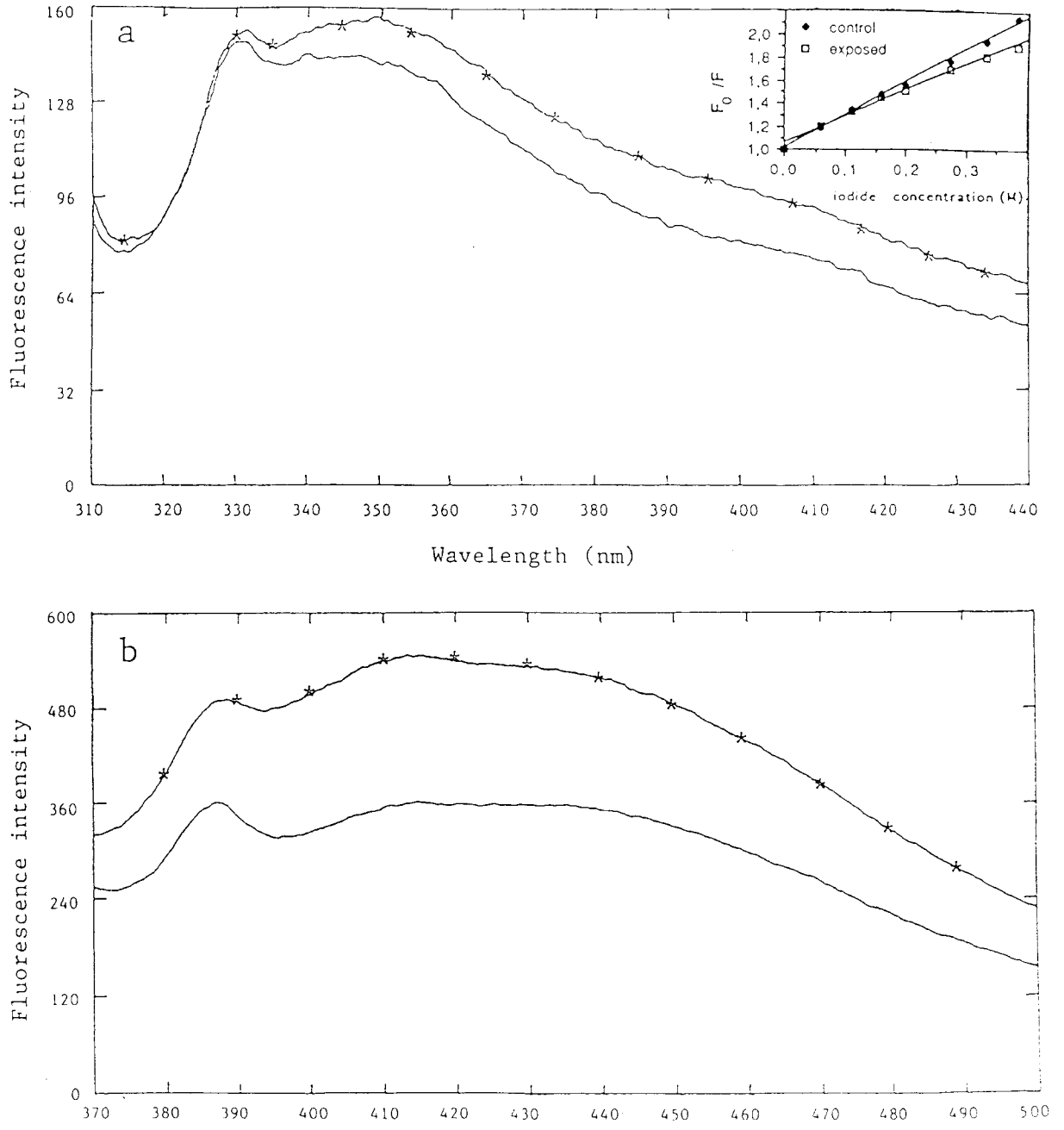

Fig. 3. Fluorescence emission spectra of irradiated AdoHcy hydrolase (*) in comparison with that of the control enzyme (solid line). Excitation was at 280 nm (a) and at 340 nm (b). The enzyme was irradiated for 1 h at 80°C (SAR, 2.0 W/g). In the inset is reported the fluorescence emission quenching of irradiated and control AdoHcy hydrolase by iodide ions. The fluorescence emission at 340 nm exciting at 280 nm was plotted against iodide concentration according to the Stern-Volmer equation.

the reduced form [24]. As reported in Fig. 3a, the irradiation of the enzyme causes an increase in the fluorescence intensity that is indicative of a changed localization of the fluorophores into the protein matrix. This was also confirmed by the fluorescence quenching experiment reported in the inset of Fig. 3a, which shows a rather different mean exposure of the protein intrinsic fluorophores before and after the irradiation. In fact, the slope in the Stern-Volmer plot decreased after the enzyme exposure to microwave irradiation. This indicates a less efficient quenching of the fluorophore(s), owing to their lower exposure to the quencher as a consequence of a conformational change.

The comparison of the fluorescence emission spectra after the excitation of the protein at 340 nm, reported in Fig. 3b, shows a net increase in the fluorescence intensity of the irradiated AdoHcy hydrolase, thus indicating a possible protein structural modification even of the NADH-binding region.

Fig. 4 shows the CD spectrum of AdoHcy hydrolase after microwave irradiation, in comparison with that of the enzyme control. Both spectra are characterized by a minimum centered at about 221 nm, and by a shoulder at 208–209 nm. These spectral features are indicative of the presence of both α-helix and β-sheet structures [32]. As can be seen, the most important difference between the two spectra is a decrease of

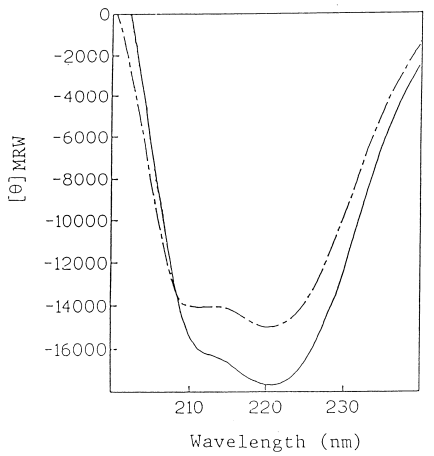

Fig. 4. Far ultraviolet CD spectra of irradiated AdoHcy hydrolase (dashed line) and control enzyme (solid line) at 25°C. The protein was irradiated for 1 h at 80°C (SAR, 2.0 W/g). Experimental tracings were accumulated and corrected for blanks by software.

the dichroic activity of irradiated AdoHcy hydrolase, thus supporting the view that microwaves bring about a structural protein rearrangement with an increase of the non-organized structure.

In conclusion, the exposure to microwave radiation causes an irreversible, time- and temperature-dependent inactivation of both enzymes. Since these enzymes are quite stable at the temperatures investigated, the results can be ascribed to non-thermal effects of microwaves.

Growing attention has been paid in recent years to potential health effects of microwave radiations. Safety standards have been set based only upon the thermal effects of microwaves [33]. The occurrence of non-thermal effects suggests that the criteria so far employed may be taken with prudence as long as the non-thermal effects of microwaves on the biomolecules are better understood.

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References

[1] Cleary, S.F. (1989) in: Electromagnetic Biointeraction (Franceschetti, G., Gandhi, O.P. and Grandolfo, M., Eds.) pp. 59–79, Plenum Press, New York.
[2] Frohlich, H. (1980) Adv. Electr. Electr. Phys. 53, 85–152.
[3] Fisun, O.I. (1993) Bioelectromagnetics 14, 55–66.
[4] Marani, E. and Feirabend, H.K.P. (1994) Eur. J. Morphol. 32, 330–334.
[5] Kirschvink, J.L. (1996) Bioelectromagnetics 17, 187–194.
[6] Taylor, L.S. (1981) Bioelectromagnetics 2, 259–267.
[7] Fesenko, E.E., Geletyuk, V.I., Kazachenko, V.N. and Chemeris, N.K. (1995) FEBS Lett. 366, 49–52.
[8] Phelan, A.M., Neubauer, C.F., Timm, R., Neirenberg, J. and Lange, D.G. (1994) Radiat. Res. 137, 52–58.
[9] Persson, B.R.R., Salford, L.G., Brun, A., Eberhard, J.L. and Malmgren, I. (1992) Ann. NY Acad. Sci. 649, 356–358.
[10] Saalman, E., Nordén, B., Arvidsson, L., Hamnerius, Y., Hoyevik, P., Connell, K.E. and Kurucsev, T. (1991) Biochim. Biophys. Acta 1064, 124- 130.
[11] Orlando, A.R., Mossa, G. and D'Inzeo, G. (1994) Bioelectromagnetics 15, 303–313.
[12] Phelan, A.M., Lange, G., Kues, H.A. and Lully, G.A. (1992) Bioelectromagnetics 13, 131–140.
[13] Bergqvist, B., Arvidsson, L., Pettersson, E., Gakt, S., Saalman, E., Hamnerius, Y. and Nordén, B. (1994) Biochim. Biophys. Acta 1201, 51–54.
[14] Marani, E. and Feirabend, H.K.P. (1993) Eur. J. Morphol. 31, 141–144.
[15] Yeargers, E.K., Langley, J.B., Sheppard, A.P. and Huddleston, J.K. (1975) Ann. NY Acad. Sci. 247, 301–304.
[16] Belkode, M.L., Johnson, D.L. and Muc, A.M. (1974) Health Phys. 26, 45–51.
[17] Bini, M., Checcucci, A., Ignesti, A., Millanta, L., Rubino, M., Camici, G., Manao, G. and Ramponi, G. (1978) J. Microwave Power Electromagn. Energy 13, 95–99.
[18] Millar, D.B., Christopher, J.P., Hunter, J. and Yeandle, S.S. (1984) Bioelectromagnetics 5, 165–172.
[19] Byus, C.V., Lundak, R.L., Fletcher, R.M. and Adey, W.R. (1984) Bioelectromagnetics 5, 341–351.
[20] Byus, C.V., Kartum, K., Pieper, S. and Adey, W.R. (1988) Cancer Res. 48, 4222–4226.
[21] d'Ambrosio, G., Massa, R., Gianfreda, L., Greco, G. Jr., and Scaglione, A. (1989) Alta Frequenza 58, 361–364.
[22] Allis, J.W. and Sinha-Robinson, B.L. (1987) Bioelectromagnetics 8, 203–212.
[23] Zappia, V., Salvatore, F., Porcelli, M. and Cacciapuoti, G. (1979) in: Biochemical and Pharmacological Roles of Adenosylmethionine and the Central Nervous System (Zappia, V., Usdin, E. and Salvatore, F., Eds.) pp. 1–16, Pergamon Press, Oxford.
[24] Porcelli, M., Cacciapuoti, G., Fusco, S., Iacomino, G., Gambacorta, A., De Rosa, M. and Zappia, V. (1993) Biochim. Biophys. Acta 1164, 79–188.
[25] Cacciapuoti, G., Porcelli, M., Bertoldo, C., De Rosa, M. and Zappia, V. (1994) J. Biol. Chem. 269, 24762–24769.
[26] Woese, C.R., Kandler, O., and Wheelis, M. (1990) Proc. Natl. Acad. Sci. USA 87, 4576–4579.
[27] d'Ambrosio, G., Lioi, M.B., Massa, R., Scarfi, M.R., Zeni, O. (1995) Electro- Magnetobiol. 14, 157–164.
[28] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[29] Porcelli, M., Cacciapuoti, G., Fusco, S., Bertoldo, C., De Rosa, M. and Zappia, V. (1996) Gene 177, 17–22.
[30] Cacciapuoti, G., Porcelli, M., Bertoldo, C., Fusco, S., De Rosa, M. and Zappia, V. (1996) Eur. J. Biochem. 239, 632–637.
[31] Arakawa, T. and Timasheff, S.N. (1982) Biochemistry 21, 6545–6552.
[32] Greenfield, N. and Fasman, J.D. (1969) Biochemistry 8, 4108–4116.
[33] Foster, K.R. and Pickard W.F. (1987) Nature 330, 531–532.