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Potentiation of thermal inactivation of glyceraldehyde-3-phosphate dehydrogenase by photodynamic treatment

A possible model for the synergistic interaction between photodynamic therapy and hyperthermia

Carla PRINSZE, Tom M. A. R. DUBBELMAN and John VAN STEVENINCK*
Sylvius Laboratories, Department of Medical Biochemistry, P.O. Box 9503, 2300 RA Leiden, The Netherlands

Thermal inactivation of glyceraldehyde-3-phosphate dehydrogenase appeared to be caused by a conformational mechanism, without involvement of covalent reactions. On the other hand, photodynamic inactivation of the enzyme (induced by illumination in the presence of Photofrin II) was caused by photo-oxidation of the essential thiol group in the active centre. A short photodynamic treatment of the enzyme, leading to only a limited inactivation, caused a pronounced potentiation of subsequent thermal inactivation, as measured over the temperature range 40–50 °C. Analysis of the experimental results according to the Arrhenius equation revealed that both the activation energy of thermal inactivation and the frequency factor (the proportionality constant) were significantly decreased by the preceding photodynamic treatment. The experimental results indicate a mechanism in which limited photodynamic treatment induced a conformational change of the protein molecule. This conformational change did not contribute to photodynamic enzyme inhibition, but was responsible for the decreased frequency factor and activation energy of subsequent thermal inactivation of the enzyme. The opposing effects of decreased activation energy and decreased frequency factor resulted in potentiation of thermal inactivation of the enzyme over the temperature range 40–50 °C. With other proteins, different results were obtained. With amylase the combined photodynamic and thermal effects were not synergistic, but additive, and photodynamic treatment had no effect on the frequency factor and the activation energy of thermal inactivation. With respect to myoglobin denaturation, the photodynamic and thermal effects were antagonistic over the whole practically applicable temperature range. Limited photodynamic treatment protected the protein against heat-induced precipitation, concomitantly increasing both the frequency factor and the activation energy of the process. These results offer a model for one of the possible mechanisms of synergistic interaction between photodynamic therapy and hyperthermia in cancer treatment.

INTRODUCTION

Photodynamic therapy (PDT) is the treatment of malignant lesions with visible light after systematic administration of a tumour-localizing photosensitizer. The drug currently used in clinical PDT is Photofrin II. The sensitizer interacts with visible light by both type I and type II photochemical pathways [1]. Most PDT-induced damage is caused by singlet oxygen, generated via the type II mechanism, but the type I, radical-mediated, mechanism will presumably contribute to the ultimate effect [2–4]. Photodynamic cytotoxicity is caused by photo-oxidation of proteins, lipids and nucleic acids [5–9].

Hyperthermia is another modality in cancer therapy, frequently used in combination with other treatments [10,11]. Although the actual mechanism of heat-induced cell death is unknown, the high activation energy of hyperthermic cell killing suggests the involvement of structural or enzymic proteins rather than of, e.g., DNA molecules [12–14]. Recently a synergistic interaction between PDT and hyperthermia has been described both in an experimental mouse tumour system [15,16] and in cells in culture [17], but the background of this synergism is quite unknown.

Many different mechanisms may result in synergism at the cellular level. For instance, the synergistic interaction of PDT and hyperthermia might be the result of a concerted action of both treatments on the same cellular component [13], or one treatment might reduce the cellular ability to repair the damage inflicted by the other [17]. Despite this complexity, elucidation of the various mechanisms of synergism is crucial to optimize therapeutic protocols. The present investigations were designed to study one of the possible mechanisms of synergistic interaction between PDT and hyperthermia, namely the concerted action on the same cellular target. As proteins are potential targets of both PDT and hyperthermia, the combined effects of photodynamic treatment and of heat on some enzymes were studied in detail. Three structurally unrelated proteins (namely GAPDH, amylase and myoglobin) were utilized in an attempt to elucidate general mechanisms of interaction between the two treatments. Moreover, the mechanism of either photodynamic or heat-induced inactivation of the utilized proteins has been studied previously, facilitating interpretation of the experimental results. The results obtained were consistent with a model in which photodynamic treatment affects the conformational characteristics of a protein molecule, thus changing the susceptibility of the protein to subsequent hyperthermia. Under appropriate conditions this can lead to a synergistic interaction between PDT and hyperthermia on protein structure and function.

MATERIALS AND METHODS

GAPDH isolated from human erythrocytes and from *Bacillus stearothermophilus* and α-amylase (type II-A) were obtained from Sigma, and GAPDH isolated from rabbit muscle from

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDT, photodynamic therapy; DPBS, Dulbecco's phosphate-buffered saline.

* To whom correspondence should be addressed.

Boehringer Mannheim. Myoglobin, isolated from equine muscle, was purchased from Serva. N.C.T.C. mouse fibroblasts, clone L929, A.T.C.C. number CCL 1, were obtained from Flow Laboratories. Tissue-culture products and newborn-calf serum were purchased from Gibco. Photofrin II was from Photofrin Medical, Raritan, NJ, U.S.A. All other chemicals were of analytical grade and used without further purification.

To study erythrocyte-membrane-bound GAPDH, unsealed ghosts were prepared as described by Dodge *et al.* [18] with the modification described by McDaniel *et al.* [19]. Briefly, freshly drawn heparinized human blood was centrifuged and washed three times in buffered iso-osmotic NaCl and subsequently haemolysed with 9 vol. of 5 mM-sodium phosphate/1 mM-EDTA/1 mM-dithiothreitol, pH 7.5. The ghosts were pelleted by centrifugation at 37000 *g* for 20 min and washed four times in the same buffer. All operations were carried out at 4 °C. Ghosts were stored at −20 °C and used within 1 week of their preparation. As GAPDH is eluted from the membrane at high ionic strength, the ghosts were suspended in 5 mM-triethanolamine/HCl/1 mM-EDTA, pH 7.6, immediately before use, at a final concentration of 1 mg of protein/ml. The other GAPDH preparations were dissolved in a solution containing 0.1 M-triethanolamine/HCl/1.5 mM-EDTA/3 mM-$MgSO_4$, pH 7.6, at a final concentration of 50 μg/ml. Amylase was dissolved in 10 mM-sodium phosphate/1 mM-$CaCl_2$, pH 6.9, at a final concentration of 0.1 mg/ml, myoglobin in 10 mM-sodium phosphate/1 mM-$CaCl_2$, pH 8, at a final concentration of 4 mg/ml. The solutions were incubated for 5 min with Photofrin II at room temperature in the dark. The final Photofrin II concentration was 2.5 μg/ml for all GAPDH preparations, 25 μg/ml for amylase and 50 μg/ml for myoglobin.

Illumination of the solutions was performed with a standard Rollei slide projector, equipped with a 150 W quartz/halogen light bulb and a heat filter, at 300 $W \cdot m^{-2}$ (white light). The temperature in the reaction vessel remained constant (22 °C) during illumination. Heat treatment was done in a temperature-controlled waterbath (accuracy better than 0.1 °C). The samples reached the desired temperature within 3 min.

Incubation of GAPDH with H_2O_2 was performed in the same buffer supplemented with 5 μM-H_2O_2.

L929 cells were cultured, incubated with Photofrin II (5 μg/ml) and illuminated as described previously [20]. Hyperthermic treatment of the cells was performed by placing a culture dish, containing a confluent cell layer covered with 2 ml of Dulbecco's phosphate-buffered saline (DPBS), in a temperature-controlled waterbath. For determination of the GAPDH activity in L929 cells, the cells were scraped off with a rubber policeman in DPBS and disrupted by sonification at room temperature with a Branson sonifier (50 kHz) for 15 s.

GAPDH activity was assayed as described by Wu & Racker [21], and amylase activity was measured by the method of Rick & Stegbauer [22]. Thermal denaturation of myoglobin leads to precipitation of this protein. Therefore the fraction of myoglobin remaining in solution after heat treatment was taken as a measure of the native protein. Protein was determined by the Lowry method [23], thiol groups as described by Sedlak & Lindsay [24] and deamidation of asparagine and/or glutamine residues by the method of Kun & Kearney [25]. SDS/PAGE was performed by the method of Studier [26], with slight modifications [27].

RESULTS

In control experiments it appeared that illumination of the protein solutions in the absence of Photofrin II or incubation with Photofrin II in the dark had no effect on enzyme activity and did not affect the kinetics of thermal inactivation.

Photodynamically and heat-induced inactivation of GAPDH

The inactivation of rabbit GAPDH by photodynamic treatment and by hyperthermia is shown in Fig. 1. The combined effects of photodynamic treatment and hyperthermia depended on the sequence of the two treatments. With exposure to heat followed by photodynamic treatment, the effects were additive (Fig. 2*a*). In the reverse sequence, namely photodynamic treatment followed by hyperthermia, the effects were clearly synergistic (Fig. 2*b*). After an illumination time of 4 min, for instance, the enzyme activity was decreased to 84% of the control value. During subsequent heat treatment this residual enzyme activity (normalized to 100% activity in Fig. 2) decreased to 52% after a heat treatment of 6 min, whereas the activity of the native enzyme decreased much less, namely to 85% after the same period of hyperthermia. Similar results were obtained with human GAPDH (membrane-bound or solubilized), GAPDH from *B. stearothermophilus* and GAPDH in intact L929 fibroblasts (results not shown).

GAPDH is protected against photodynamic inactivation by its substrate, glyceraldehyde 3-phosphate [28]. A similar protection

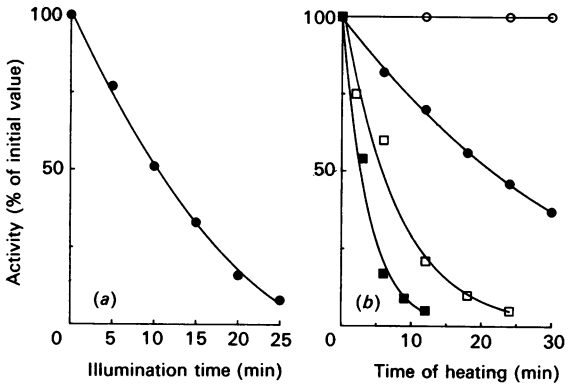

Fig. 1. Photodynamically and thermally induced inactivation of rabbit GAPDH

(*a*) Photodynamic treatment, as described in the Materials and methods section. (*b*) Heat treatment at: ○, 20 °C (control); ●, 43 °C; □, 46 °C; ■, 48 °C.

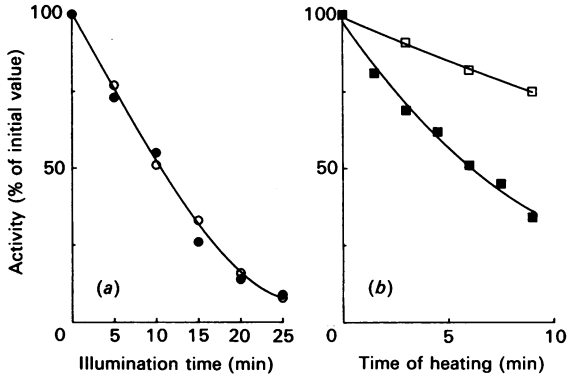

Fig. 2. Effect of photodynamic treatment, hyperthermia and a combination of these treatments on the activity of rabbit GAPDH.

(*a*): ○, Photodynamic treatment of the native enzyme; ●, enzyme exposed to 43 °C for 5 min (residual enzyme activity 80% of control, normalized to 100% in the Figure), followed by photodynamic treatment. (*b*): □, exposure of native enzyme to 43 °C; ■, 4 min of photodynamic treatment (residual enzyme activity 84% of control, normalized to 100% in the Figure), followed by exposure to 43 °C.

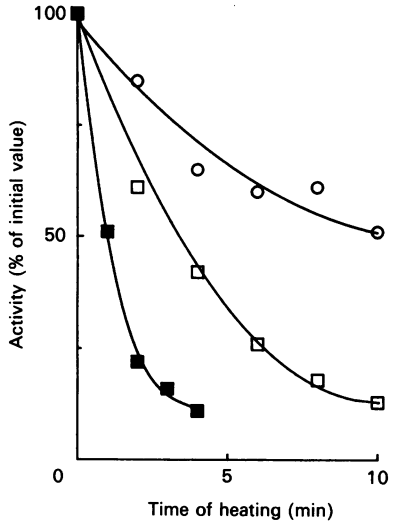

Fig. 3. Heat-induced inactivation of rabbit GAPDH in the presence of 2 mM-ATP, 12 mM-glycerate 3-phosphate and phosphoglycerate kinase (30 units/ml), with and without preceding photodynamic treatment

○, Exposure of native enzyme to 58 °C; □, 30 min of photodynamic treatment (yielding no measurable enzyme inhibition), followed by exposure to 58 °C; ■, 60 min of photodynamic treatment (residual enzyme activity 76% of control, normalized to 100% in the Figure), followed by exposure to 58 °C.

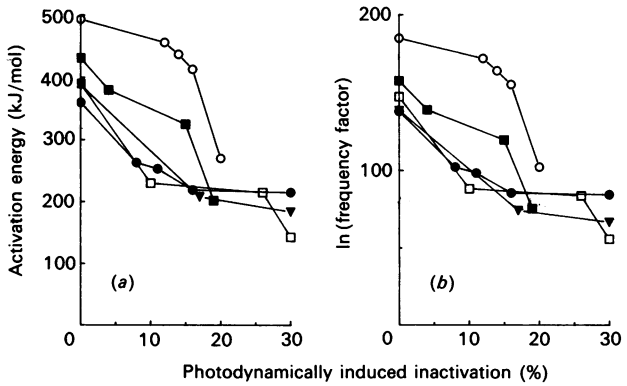

Fig. 4. Influence of photodynamic pretreatment on the Arrhenius parameters of thermal inactivation of GAPDH

Photodynamic treatment was performed with various illumination periods, leading to enzyme inactivations of 0–30%, as indicated on the ordinate. (*a*) Activation energy; (*b*) frequency factor (expressed as *A*). ●, Rabbit GAPDH; ○, solubilized human GAPDH; ■, membrane-bound human GAPDH; ▼, GAPDH from *B. stearothermophilis*; □, GAPDH in intact L929 fibroblasts.

against heat-induced inactivation of the enzyme was observed in the present studies. Figs. 1(*b*) and 3 demonstrate that the velocity of inactivation at 58 °C in the presence of substate is about equal to the velocity of inactivation at 43 °C in its absence. Also in the presence of substrate photodynamic treatment strongly potentiated subsequent heat inactivation of the enzyme, as shown in Fig. 3.

Photodynamic effect on the activation energy and frequency factor of thermal inactivation

Analysis of the potentiation of thermal inactivation of GAPDH by photodynamic pre-treatment revealed that the

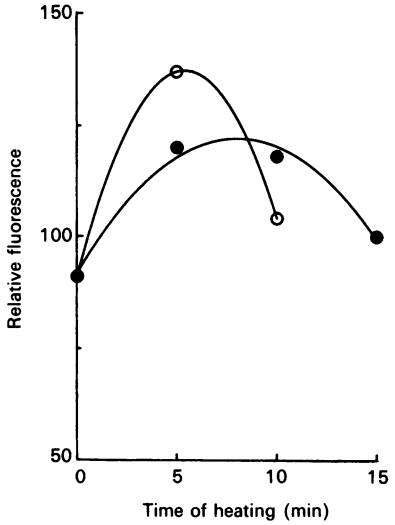

Fig. 5. Thermally induced changes of intrinsic fluorescence of rabbit GAPDH (excitation 280 nm, emission 340 nm)

○, 50 °C; ●, 46 °C.

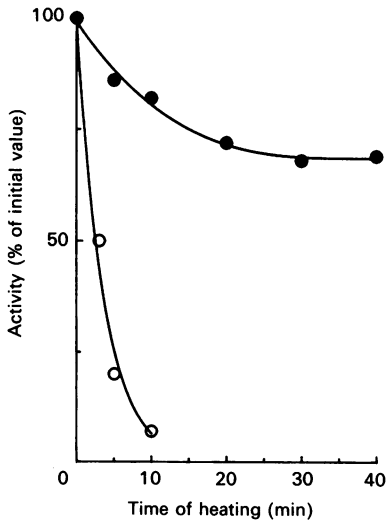

Fig. 6. Thermal inactivation of rabbit GAPDH at 48 °C in the absence (○) and in the presence (●) of 25% glycerol

magnitude of the effect depended on the temperature during heat exposure. For instance, with a photodynamic treatment resulting in 16% inhibition of the enzyme activity, the velocity of subsequent heat inactivation of the residual enzyme activity at 40 °C was about 2.2 times the velocity of inactivation of the native enzyme. At 45 °C this factor was decreased to 1.4, and at 50 °C the potentiating effect had virtually disappeared. At higher temperatures the velocity of inactivation was too high to allow accurate measurements. Considering these observations, both the activation energy and the frequency factor of thermal inactivation were calculated under various experimental conditions, utilizing the Arrhenius equation:

$$\ln k = \ln A - E/RT,$$

in which k is the reaction constant, A is the frequency factor, E is the activation energy, R is the gas constant and T is the temperature. The activation energy of thermal inactivation of

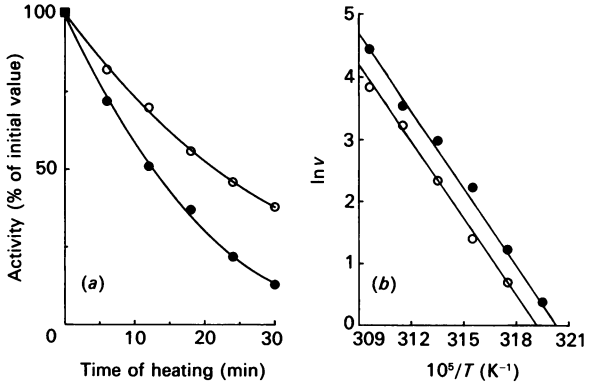

Fig. 7. Effect of pre-incubation of rabbit GAPDH for 60 min with 5 μM-H_2O_2 (residual enzyme activity 71% of control, normalized to 100% in the Figure) on subsequent thermal inactivation at 43 °C and (*b*) Arrhenius plot of thermal inactivation of native GAPDH (○) and of GAPDH, preincubated for 60 min with 5 μM-H_2O_2 (●)

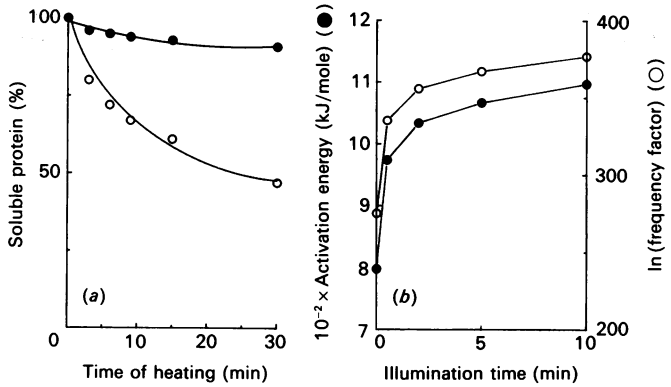

Fig. 8. Influence of photodynamic pre-treatment on heat-induced precipitation of myoglobin

(*a*) Protein precipitation during exposure to 78 °C. ○, Native myoglobin; ●, myoglobin, illuminated during 30 min in the presence of 50 μg of photofrin II/ml before heat treatment. (*b*) The effect of photodynamic treatment on the activation energy (●) and the frequency factor (○) of subsequent thermal denaturation.

rabbit GAPDH decreased significantly with preceding photodynamic treatment, with a concomitant decrease of the frequency factor (Fig. 4). Also, with solubilized GAPDH from several other sources, erythrocyte-membrane-bound GAPDH and GAPDH in intact L929 fibroblasts, a decrease of both the activation energy and the frequency factor was found, but the shifts differed markedly with the different enzyme preparations (Fig. 4).

Effects of hyperthermia on GAPDH at the molecular level

As thermal inactivation of enzymes may be caused by several different mechanisms, additional studies were performed. Measurements on native GAPDH and on the enzyme after complete inactivation by exposure to 46 °C during 20 min revealed no change of the number of thiol groups and no NH_4^+ generation after heat treatment, indicating that no deamidation had occurred. Further, no fragmentation of the enzyme molecule was found, on the basis of SDS/PAGE subsequent to heat-treatment (results not shown), excluding appreciable hydrolysis of peptide bonds. Heat treatment of GAPDH caused a change of the intrinsic fluorescence of the protein, as shown in Fig. 5. Finally, thermal inactivation of GAPDH was strongly suppressed by the presence of 25% glycerol (Fig. 6).

Potentiation of thermal inactivation of GAPDH by H_2O_2

The pronounced potentiation of thermal inactivation of GAPDH by photodynamic treatment is apparently not a specific photodynamic effect. Fig. 7(*a*) illustrates a similar potentiation by pretreatment with low concentrations of H_2O_2. Kinetic analysis of the results obtained at different temperatures indicated that, after exposure to H_2O_2, the activation energy of thermal inactivation remained constant within the experimental errors, whereas the frequency factor was increased (Fig. 7*b*). Inactivation of GAPDH by H_2O_2 alone is strictly related to modification of thiol groups [29], leading to the generation of disulphides and sulphenic acid residues [29,30].

Combined photodynamic and heat effects on other proteins

The potentiation of thermal inactivation by preceding photodynamic treatment is not a general phenomenon, as demonstrated by the following experiments. Amylase was inactivated both by photodynamic and by heat treatment. In contrast with the results obtained with GAPDH, however, preceding photodynamic treatment did not potentiate subsequent heat inactivation. Instead the two effects were additive. More detailed analysis revealed that preceding photodynamic treatment (up to an enzyme inactivation of 30%), did not affect the activation energy of thermal inactivation (266 kJ/mol), nor the frequency factor (exp 104.5; results not shown).

Heat treatment leads to aggregation and precipitation of myoglobin (Fig. 8), whereas the solubility of myoglobin is not affected by photodynamic treatment. Surprisingly, photodynamic treatment protected the protein from subsequent heat-induced precipitation (Fig. 8*a*), with a concomitant increase of both the activation energy and the frequency factor of heat-induced precipitation, in an illumination-time-dependent fashion (Fig. 8*b*). These effects were not observed after illumination in the absence of Photofrin II or after incubation with Photofrin II in the dark.

DISCUSSION

Thermoinactivation of enzymes may be caused by a conformational process (with formation of incorrect, scrambled structures on cooling), modification of thiol groups, deamidation of asparagine and/or glutamine residues, hydrolysis of peptide bonds, or any combination of these processes [31–33]. In the case of thermal inactivation of GAPDH the results described rule out the possibility that thiol modification, deamidation and peptide hydrolysis would play a significant role in thermal inactivation, and thus it is highly likely that the inactivation is a conformational process. A conformational rather then a covalent mechanism of thermal inactivation of GAPDH is sustained by: (1) the rapid inactivation at near-neutral pH at temperatures well below 70 °C (Fig. 1*b*) [33]; (2) the high activation energy of the process (about 360 kJ/mol) [12–14;31–33]; (3) the protection against thermal inactivation by the substrate of the enzyme (Figs. 1*b* and 3) [32,33]; (4) the protection afforded by glycerol (Fig. 6) [14,34,35]; and (5) the changed intrinsic fluorescence of the enzyme during heat treatment (Fig. 5), which is a very sensitive indicator of conformational changes [33,36–38].

In a previous paper it was demonstrated that even very limited

photodynamically or OH· (hydroxyl radical)-induced protein damage caused pronounced conformational changes [39]. Photodynamic inactivation of GAPDH was strictly related to the photo-oxidation of the essential thiol group in the active centre, however, without appreciable contribution of the conformational changes to inactivation [28,39]. It is also unlikely that such conformational changes would affect thermally induced covalent reactions, as the rates of these reactions are similar in structurally different proteins [32,33]. However, as these photodynamically induced lesions clearly influence the structural stability of the protein [39], they might easily affect the susceptibility of the protein to thermally induced conformational deterioration. In this context it should be realized that photodynamic treatment of proteins does not only lead to photo-oxidation of susceptible amino acid residues, but also to intramolecular cross-links [40,41]. It was shown previously that intramolecular crosslinks, generated via quite different mechanisms, may either increase or decrease the susceptibility of different enzymes to thermal inactivation [42,43]. It seems highly probable that photodynamically generated intramolecular cross-links will, in a similar way, affect the susceptibility of proteins to thermally induced conformational changes. The magnitude and direction of these effects cannot be predicted, however, as shown by the present results. With GAPDH, photodynamic treatment potentiated subsequent thermal inactivation over the temperature range 40–50 °C (Figs. 2*b* and 7). With amylase, on the other hand, the photodynamic and heat effects were additive, and with myoglobin an antagonistic effect was found (Fig. 8).

In all cases the potentiating, additive or antagonistic effect of photodynamic pretreatment on subsequent thermal denaturation could be described in terms of the Arrhenius equation. For instance, photodynamic treatment of GAPDH caused a decrease of the activation energy of thermal inactivation, tending to potentiate this process. The concomitant decrease of the frequency factor, however, opposed this effect. Together this resulted in a potentiation at temperatures below 50 °C.

The effect of conformational changes on the activation energy of thermoinactivation is well-known [44]. Further, according to the transition-state theory, the frequency factor in the Arrhenius equation in complex reactions is equal to $\boldsymbol{RT}/Nh \cdot \exp(\Delta S/\boldsymbol{R})$ [45], in which N is Avogadro's number, h is Planck's constant, S is the standard entropy of formation of the transition state and $\boldsymbol{R}$ and T have their usual meanings. As denaturation of proteins is a complicated and continuous multi-step process [46], the frequency factor will be even more complex and only constant over a limited temperature range. It is likely that both photo-oxidation of susceptible amino acid residues and photodynamically induced intramolecular crosslinks will affect ΔS and thus the frequency factor of thermoinactivation. Considering the unpredictable effect of photodynamic treatment on the activation energy and the frequency factor of subsequent thermal inactivation, it is clear from the Arrhenius equation that the combined effects of the two treatments may be additive, synergistic or antagonistic, depending on the experimental conditions. In fact it is even feasible that synergism can be observed at a particular temperature and antagonism at another.

As mentioned in the Introduction, a synergistic interaction between photodynamic therapy and hyperthermia has been described on a cellular and at an '*in vivo*' level [15–17]. A concerted action of two treatments on the same cellular target is only one of several mechanisms that may result in synergism [13,17]. The results presented here describe a possible mechanism for this particular type of synergism, in which photodynamic treatment causes conformational changes in a protein molecule, thus affecting the susceptibility to subsequent thermal deterioration.

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REFERENCES

1. Foote, C. S. (1984) in Porphyrin Localization and Treatment of Tumors (Doiron, D. R. & Gomer, C. G., eds.), pp. 301–334, A. R. Liss, New York
2. Van Steveninck, J., Tijssen, K., Boegheim, J. P. J., Van der Zee, J. & Dubbelman, T. M. A. R. (1986) Photochem. Photobiol. **44**, 711–716
3. Hariharan, P. V., Courtney, J. & Eleczko, S. (1980) Int. J. Radiat. Biol. **37**, 691–694
4. Das, M., Mukhtar, H., Greenspan, E. R. & Bickers, D. R. (1985) Cancer Res. **45**, 6328–6330
5. Roberts, J. E., Roy, D. & Dillon, J. (1985) Curr. Eye Res. **4**, 181–185
6. Schothorst, A. A., Van Steveninck, J., Went, L. N. & Suurmond, D. (1972) Clin. Chim. Acta **39**, 161–170
7. Piette, J., Merville-Louis, M. P. & Decuyper, J. (1986) Photochem. Photobiol. **44**, 793–802
8. Dubbelman, T. M. A. R., Van Steveninck, A. L. & Van Steveninck, J. (1982) Biochim. Biophys. Acta **719**, 47–52
9. Doleiden, F. H., Fahrenholtz, S. R., Lamola, A. A. & Trozzola, A. M. (1974) Photochem. Photobiol. **20**, 519–521
10. Gerweck, L. E. (1985) Cancer Res. **45**, 3408–3414
11. Dewey, W. C. (1984) Cancer Res. **44**, 4714s–4720s
12. Jorritsma, J. B. M., Burgman, P., Kampinga, H. H. & Konings, A. W. T. (1986) Radiat. Res. **105**, 307–319
13. Leyko, W. & Bartosz, G. (1986) Int. J. Radiat. Biol. **49**, 743–770
14. Dewey, W. C. (1989) Radiat. Res. **120**, 191–204
15. Henderson, B. W., Waldow, S. M., Potter, W. R. & Dougherty, T. J. (1985) Cancer Res. **45**, 6071–6077
16. Mang, T. S. & Dougherty, T. J. (1985) Photochem. Photobiol. **42**, 533–540
17. Christensen, T., Wahl, A. & Smedshammer, L. (1984) Br. J. Cancer **50**, 85–89
18. Dodge, J. T., Mitchell, C. & Hanaman, D. J. (1963) Arch. Biochem. Biophys. **100**, 119–130
19. McDaniel, C. F., Kirtley, M. E. & Tanner, M. J. A. (1974) J. Biol. Chem. **249**, 6478–6485
20. Dubbelman, T. M. A. R. & Van Steveninck, J. (1984) Biochim. Biophys. Acta **771**, 201–207
21. Wu, R. & Racker, E. (1959) J. Biol. Chem. **234**, 1029–1035
22. Rick, W. & Stegbauer, H. P. (1974) in Methods in Enzymatic Analysis (Bergmeyer, H. U., ed.), vol. 2, pp. 885–890, Academic Press, New York
23. Lowry, D. H., Rosebrough, N. J., Farr, A. L. & Randall, R. I. (1951) J. Biol. Chem. **193**, 265–270
24. Sedlak, J. & Lindsay, R. H. (1968) Anal. Biochem. **25**, 195–205
25. Kun, E. & Kearney, E. B. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), 2nd edn., vol. 4, pp. 1802–1806, Academic Press, New York
26. Studier, F. W. (1973) J. Mol. Biol. **79**, 237–248
27. Yamada, K. H. & Weston, J. A. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 3492–3496
28. Dubbelman, T. M. A. R., De Goeij, A. F. P. M. & Van Steveninck, J. (1978) Photochem. Photobiol. **28**, 197–204
29. Little, C. & O'Brien, P. J. (1969). Eur. J. Biochem. **10**, 533–538
30. Means, G. E. & Feeney, R. E. (1971). Chemical Modification of Proteins, pp. 162–165, Holden-Day, San Francisco
31. Tomazic, S. J. & Klibanov, A. M. (1988) J. Biol. Chem. **263**, 3092–3096
32. Tomazic, S. J. & Klibanov, A. M. (1988) J. Biol. Chem. **263**, 3086–3091
33. Ahern, T. J. & Klibanov, A. M. (1987) Methods Biochem. Anal. **33**, 91–127
34. Mivechi, N. F. & Dewey, W. C. (1984) Radiat. Res. **99**, 352–362
35. Back, J. F., Oakenfull, D. & Smith, M. B. (1979) Biochemistry **18**, 5191–5196
36. Wasylewski, Z., Criscimagna, N. L. & Horowitz, P. M. (1985) Biochim. Biophys. Acta **831**, 201–206
37. Mantulin, W. W., Rohde, M. F., Gotto, A. M. & Pownall, H. J. (1980) J. Biol. Chem. **255**, 8185–8191
38. Hopkins, T. R. & Spikes, J. D. (1970) Photochem. Photobiol. **12**, 175–184

39. Prinsze, C., Dubbelman, T. M. A. R. & Van Steveninck, J. (1990) Biochim. Biophys. Acta **1038**, 152–157
40. Verweij, H. & Van Steveninck, J. (1982) Photochem. Photobiol. **35**, 265–267
41. Van Steveninck, J. & Dubbelman, T. M. A. R. (1984) Biochim. Biophys. Acta **791**, 98–101
42. Klemes, Y. & Citri, N. (1980) Biochem. J. **187**, 529–532
43. Arnold, L. D. & Viswanatha, T. (1983) Biochim. Biophys. Acta **749**, 192–197
44. Violet, M. & Meunier, J. C. (1989) Biochem. J. **263**, 665–670
45. Latham, J. L. (1962) Elementary Reaction Kinetics, pp. 42–53, Butterworths, London
46. Malhotra, O. P. & Srinivasan (1985) Arch. Biochem. Biophys. **236**, 775–781

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