Modification of Thermal Response of *Chlorella ellipsoidea* by Deuteration

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The isotope effect of deuterium (D) on cellular thermo-response, one of the major cell functions, was pursued by using deuterated *Chlorella ellipsoidea* which was grown in D_2O medium and deuterated proportionally to the concentration of D_2O in the medium. The deuterated *Chlorella* (D-*Chlorella*) cells heat-treated at 43 °C in H_2O medium were more heat-sensitive than control *Chlorella* (H-*Chlorella*) cultured in H_2O medium. The D-*Chlorella* cells grown in higher mol% D_2O were more heat-sensitive than those grown in lower mol% D_2O. On the other hand, both the D- and H-*Chlorella* cells heat-treated at 43 °C in D_2O medium were more heat-resistant than in H_2O medium. The degree of heat resistance was linearly proportional to the concentration of D_2O in the medium. These results suggest that D in the medium and cells had two opposite effects on *Chlorella*; deuteration of poorly exchangeable regions of biomolecules in the cells made *Chlorella* heat-sensitive, and both D_2O in the medium and D in easily exchangeable regions caused *Chlorella* to be heat-resistant.

Keywords deuteration; thermal response; *Chlorella ellipsoidea*; deuterium oxide; isotope effect; heat sensitivity; heat resistance; constitutional isotope effect; solvent isotope effect

A number of studies on the biological isotope effect of deuterium (D) have pointed out that intra- and extracellular D affect several functions of cells in a variety of organisms.[1-5] However, the mechanisms involved have not been fully elucidated. Concerning the isotope effects of D on cell functions, it has been indicated that cellular macromolecules such as proteins and nucleic acids are stabilized to heat by D_2O,[6-9] suggesting that D_2O may alter the thermal response of cells. Actually, several studies have demonstrated that the cell-killing temperature was altered by the presence of D_2O.[10-13] However, the data were not always consistent; the alteration of heat sensitivity by D_2O, either decrease or increase, depended on the experimental conditions.[11-13]

These inconsistencies might have arisen from failure to distinguish in the analysis between different isotope effects due to D_2O in medium and cell-constitutional D; the latter may be further classified into easily exchangeable D (exchangeable D) and poorly exchangeable D (unexchangeable D) of macromolecules in cells.[14] To distinguish these effects we have utilized deuterated *Chlorella* (D-*Chlorella*) which was obtained by culturing *Chlorella* in D_2O medium and of which the D content was proportional to the molar ratio of D_2O in the medium.[14]

The present paper describes the differential isotope effects of D_2O in the medium and of cell-constitutional D on the thermal response of *Chlorella*; temperature is one of the most critical environmental factors for cells and the heat-protecting response is one of the most important functions of cells in all organisms, as suggested by recent studies on heat-shock proteins.[15,16] Here, we found that the thermal responses of *Chlorella* in D_2O and D-*Chlorella* in H_2O were opposite; the former became heat-resistant and the latter heat-sensitive.

Materials and Methods

Cultivation of D-*Chlorella* The cells of *Chlorella ellipsoidea* GERNECK (C-27) were cultured in Myers 4N (M-4N) medium containing 0—100 mol% D_2O[14] at 25 °C under 18 klx light and with continuous bubbling of dry air containing 5% CO_2. To obtain fully deuterated cells, cultivation in D_2O medium was performed for more than 10 d. The cells were deuterated proportionally to the concentration of D_2O in the medium.[14]

Heat Treatment The exponentially growing *Chlorella* cells cultured at 25 °C were collected by centrifugation and resuspended in H_2O or D_2O medium. The cell suspension (1×10^7 cells/ml) was heat-treated at 30—45 °C in a water-bath shaker (Taiyo Sci. Ind.), and then rapidly returned to 25 °C. The temperature for the heat treatment was maintained within ±0.1 °C. The cells thus treated were cultured in 5 ml of H_2O medium at 25 °C for 4—7 d. The growth ratio of the heat-treated group to the untreated (25 °C) control was calculated from the absorbance at 660 nm of each group at the second or third day of cultivation, when cells were growing exponentially. The 50% growth inhibition temperatures of H- and D-*Chlorella* were obtained from the curves of each growth ratio at the third day.

Survival Assay Agar plates were prepared as follows; 15 mg of KH_2PO_4, 15 mg of $MgSO_4 \cdot 7H_2O$, 0.2 mg of $FeSO_4 \cdot 7H_2O$, 100 mg of $Ca(NO_3)_2 \cdot 4H_2O$, 25 mg of KCl and 1.5 g of agar in 100 ml of H_2O. H- or D-*Chlorella* cells (1×10^2 cells) heat-treated as described above were inoculated on a plate and cultured at 25 °C for 10 d under 10 klx light. The values of survival fractions were normalized to that of the heat-untreated (25 °C) control for each of H- and D-*Chlorella*, and the 50% survival temperature was determined for each *Chlorella*.

Results

Response of H- and D-*Chlorella* to Heat Treatment in H_2O Medium *Chlorella* cells cultured in 60 mol% D_2O (60% D-*Chlorella*) and H-*Chlorella* were suspended in H_2O medium and heat-treated at 43 °C for 0—2 h. Thereafter, those cells were cultured in H_2O medium for 4 d and their growth was determined by measuring the absorbance at 660 nm. The results showed that the growth of both the H- and D-*Chlorella* was suppressed proportionally to the time of heat treatment (Fig. 1). When D-*Chlorella* was compared with H-*Chlorella*, the former was more heat-sensitive than the latter, suggesting that the deuteration of unexchangeable regions of cell constituents made *Chlorella* thermosensitive.

Heat Sensitivity of D-*Chlorella* in H_2O Medium To examine the effect of cell-constitutional D on the heat sensitivity of *Chlorella*, D-*Chlorella* cells cultured in 60—90 mol% D_2O media were heat-treated at 43 °C for 1 h. D-*Chlorella* cultured in higher concentrations of D_2O was found to be more heat-sensitive (Fig. 2). Because D content in cells was proportional to the concentration of D_2O in the medium and because, on transferring those cells into H_2O medium, exchangeable D in cells was rapidly exchanged by H,[14] it was suggested that the heat sensitivity of *Chlorella*

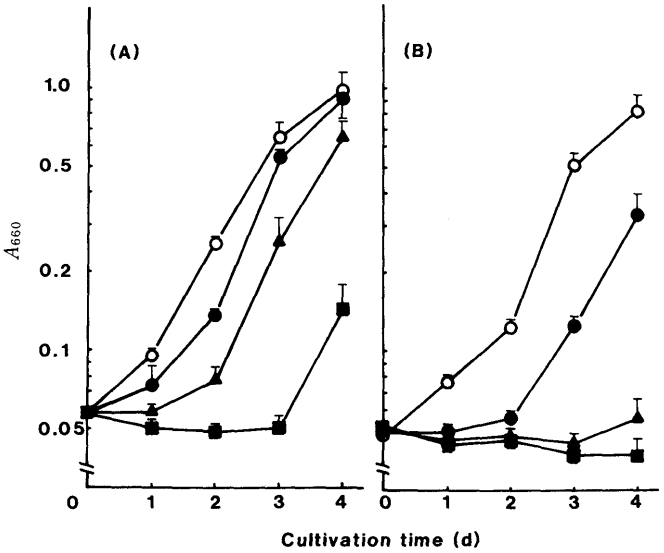

Fig. 1. Response of *Chlorella* to Heat Treatment in H_2O Medium

H-*Chlorella* (A) and 60% D-*Chlorella* (B) were heat-treated at 43 °C for 0 h (○), 0.5 (●), 1.0 (▲), and 2.0 h (■) in H_2O medium (1×10^7 cells/ml). The heat-treated cell suspensions were immediately returned to 25 °C after the treatments, and photoautotrophically cultured in 5 ml of H_2O medium. Each point and bar represent the mean ± S.E. ($n=3$).

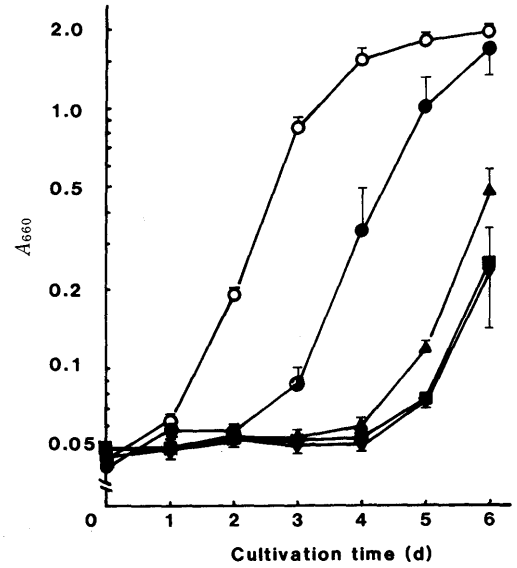

Fig. 2. Heat Sensitivity of D-*Chlorella* in H_2O Medium

60—90% D-*Chlorella* cells in H_2O medium were heat-treated at 43 °C for 1 h in H_2O medium, and then cultured at 25 °C. The growth curves, shown by absorbance at 660 nm, of *Chlorella* are represented as follows; 60% D-*Chlorella* (●), 70% D-*Chlorella* (▲), 80% D-*Chlorella* (■) and 90% D-*Chlorella* (▼). 60% D-*Chlorella* unheated and cultured at 25 °C in H_2O medium is shown as the control (○). Each point and bar represent the mean ± S.E. ($n=3$).

increased proportionally to the D content in the unexchangeable region. The increase in heat sensitivity caused by the deuteration of cell constituents was further confirmed on 0—90 mol% D-*Chlorella* by determining the 50% survival temperatures and by comparing them with their 50% growth inhibition temperatures; the former was obtained by counting the colony numbers formed on agar plates and the latter by estimating the growth ratio of heat-treated cells to untreated at the third day. Both the 50% survival temperature and the 50% growth inhibition temperature were lower in *Chlorella* containing higher D

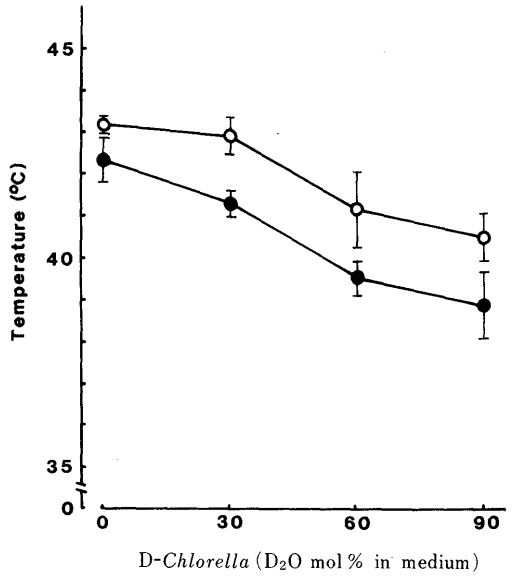

Fig. 3. Temperatures Giving 50% Survival and 50% Growth Inhibition of D-*Chlorella*

H-*Chlorella* and 30%, 60% and 90% D-*Chlorella* cells were heat-treated at 30, 37, 41, 43, or 45 °C for 1 h. The 50% survival temperature (○) of each D-*Chlorella* was obtained from the ratio of colony numbers of heated cells to unheated; the colonies were formed by photoautotrophical cultivation (10 d) of the cells (1×10^2 cells/agar plate). The 50% growth inhibition temperature (●) was obtained from the ratio (heated/unheated) of absorbances at 660 nm of the cell suspensions at the 3rd day of cultivation. Each point and bar represent the mean ± S.E. ($n=3$ or 4).

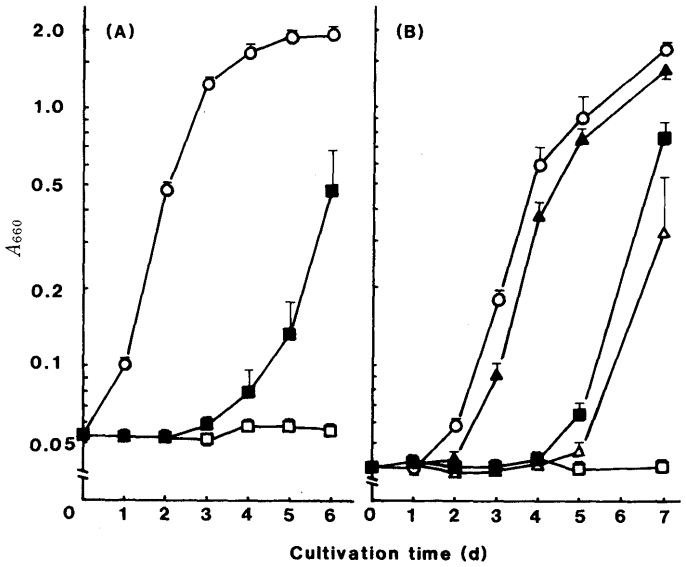

Fig. 4. Heat Response of *Chlorella* in D_2O Medium

(A) H-*Chlorella* (1×10^7 cells) was heat-treated at 43 °C for 2 h in H_2O medium (□) or 60 mol% D_2O medium (■), and then cultured in H_2O medium. Control cells (○) were similarly treated at 25 °C and then cultured.

(B) 60% D-*Chlorella* (1×10^7 cells) was heat-treated at 43 °C as follows; for 1 h in H_2O (△) or 60 mol% D_2O (▲), and for 2 h in H_2O (□) or 60 mol% D_2O (■). Control cells (○) were treated at 25 °C for 2 h in H_2O. The treated cells were then cultured in H_2O medium. Each point and bar represent the mean ± S.E. ($n=3$).

contents (Fig. 3). The 50% survival temperature and the 50% growth inhibition temperature of 90% D-*Chlorella* were 2.7 and 3.5 °C, respectively, lower than those of H-*Chlorella*. It was also found that the 50% growth inhibition temperature was in parallel with the 50% survival temperature, suggesting that the growth ratio might reflect the surviving cell number. Therefore, the measurement of the former, which was practically easier than that of the latter,

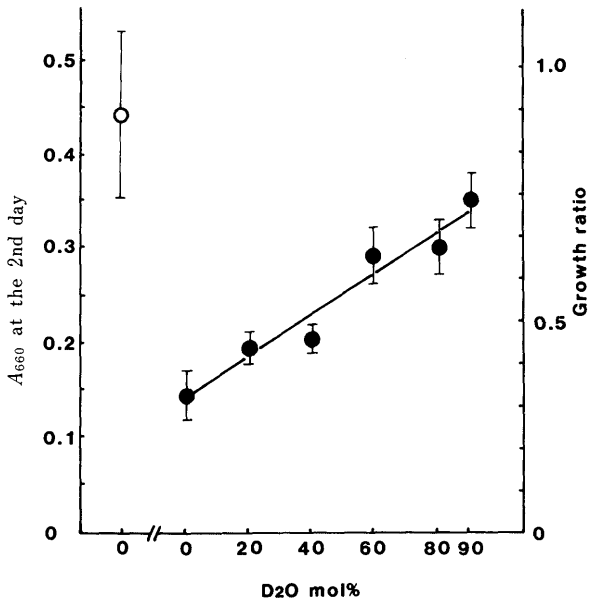

Fig. 5. Heat Resistance of H-*Chlorella* in D_2O Medium

H-*Chlorella* cells (1×10^7 cells) were heat-treated at 43 °C in 0—90 mol% D_2O medium. Absorbances at 660 nm at the 2nd day were measured for heated cells (●) and unheated (○). Growth ratio was designated by A_{660} the ratio of heated cells to unheated. Each point and bar represent the mean ± S.E. ($n=3$).

seemed to be enough to determine the heat sensitivity.

Heat Response of H- and D-*Chlorella* in D_2O Medium The heat response of *Chlorella* in D_2O was compared with that in H_2O. H-*Chlorella* cells heat-treated in 60 mol% D_2O medium at 43 °C for 2 h were found to be remarkably more heat-resistant than the cells treated in H_2O medium (Fig. 4A). Further, 60% D-*Chlorella* cells in 60 mol% D_2O medium were also much more heat-resistant than the cells in H_2O when heat-treated at 43 °C for 1 or 2 h (Fig. 4B). When H-*Chlorella* cells were heat-treated in 0—90 mol% D_2O, the cells became heat-resistant proportionally to the concentration of D_2O (Fig. 5). These results suggest that the solvent effect of D_2O and, possibly, the isotope effect of exchangeable D in cells brought about the increase in thermo resistance of *Chlorella*.

Discussion

The isotope effect of D on the heat-shock response of *Chlorella* cells was studied by a method designed to differentiate the effect of cell-constitutional D and that of D in the medium. The former was further subdivided into exchangeable D and unexchangeable D. This unexchangeable D was assayed by transferring D-*Chlorella* into H_2O medium, whereupon exchangeable D was rapidly exchanged with H.[14] On the other hand, the solvent effect of D_2O was estimated by transferring H-*Chlorella* into D_2O medium, although the effect of exchangeable D was included in this system.

By utilizing these systems, we found that D-*Chlorella* in H_2O medium was more heat-sensitive than H-*Chlorella* in H_2O; the elevation of heat sensitivity was proportional to the D content in cells (Figs. 1—3). This finding indicates that the deuteration of unexchangeable regions in cells made *Chlorella* heat-sensitive. On the other hand, when H- and D-*Chlorella* cells were heat-treated in D_2O medium, both the cells became more heat-resistant than in H_2O medium (Figs. 4, 5). Apparently this observation suggests that both the solvent effect of D_2O and the isotope effect of exchangeable D in cells caused heat resistance. These two effects are, in fact, difficult to distinguish, because the exchangeable D can be easily exchanged by D in D_2O medium, and the much higher amount of D in the medium relative to cellular exchangeable D may result in a much higher effect.

The differential isotope effects observed here suggest the differential actions between unexchangeable D and solventous D including exchangeable D. The former is considered to contribute mainly to the structural stability of cellular molecules, particularly macromolecules, and the latter, to participate in the reactivity of molecules. The cellular target of heat injury is not well established. The deuterated cell might be a useful biological system of differentially modified molecular structure and reactivity for the further elucidation of not only the biological isotope effect of D but also the mechanisms of heat response of cells.

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