Deuterium-Resistant Algal Cell Line for D Labeling of Heterotrophs Expresses Enhanced Level of Hsp60 in D₂O Medium

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Fully deuterated components from autotrophic cell lysate are useful materials for labeling of heterotrophs with deuterium. To facilitate the faster production of deuterated algal lysate, we selected a mutant *Chlorella* strain that grows faster in heavy water than the wild type. The mutant DR-17 was found to have a higher level of Hsp60 and an elevated level of protein synthesis. We previously isolated a deuterium-resistant yeast cell line that was also found to express elevated level of Hsp70 (K. Unno, T. Kishido, M. Morioka, S. Okada, and N. Oku, Biol. Pharm. Bull. 26:799-802, 2003). This suggests that the overexpression of heat shock proteins is required to compensate for the deuterium isotope effect.

Stable isotope labeling is an essential tool for studying the functional structure and assembly of proteins by nuclear magnetic resonance (NMR). While high deuteration levels of non-exchangeable sites in a protein are beneficial for main chain assignment, the complete exchange of D in exchangeable sites to H is required for proton detection. If amide protons involved in strong hydrogen bonds or buried inside the protein are not accessible to H in the solvent, the amides will remain in the deuterated form. Although this problem may be resolved by unfolding the protein using chemical denaturants followed by refolding in the presence of H₂O, complete exchange and refolding is not always possible.

The expression of proteins in fully deuterated algal lysate medium in 100% H₂O has recently been described (6). Using this technique, deuterated samples were uniformly protonated at their amide sites, irrespective of the solvent exchange characteristics of the folded protein. We isolated a mutant clone to facilitate the production of deuterated growth media from algal *Chlorella*.

MATERIALS AND METHODS

Preparation of mutant algal cells and cultivation in D₂O. Algal cells (*Chlorella vulgaris* Beijerinck IAM C-27) were treated with the alkylating agents N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Aldrich Chemical Co., Inc.) and methanesulfonic acid ethyl ester (EMS; Sigma Chemical Co.) at concentrations of 6.8 μ M and 0.28 M, respectively. Approximately 30% of the cells survived under these concentrations. Surviving clones that treated with either MNNG or EMS were selected on an agar plate of Myers 4N (M-4N) medium. To compare the resistance to D₂O, the clone cells were cultured at 25°C in M-4N medium prepared with 99.9% D₂O under 18 klx light and aerated with dry air (16).

Synchronized cells were prepared in H₂O medium as below. Mature cells were divided into small daughter cells in darkness. Immature cells undivided were removed by centrifugation at 150 $\times$ g for 2 min. The daughter cells cyclically repeated the maturation and cell division in the light and dark (7, 17). The synchronized daughter cells obtained were cultured in D₂O medium. The lag phase of the cells was measured as the needed time for the first cell division of the daughter cells.

DNA, RNA, and protein synthesis in *Chlorella*. Wild-type (wt) cells (5×10^8 cells/1.5 ml) were incubated in the presence of [2-¹⁴C]thymidine, [2-¹⁴C]uridine, or ¹⁴C- α aminoisobutyric acid (α -AIB), which is an unavailable amino acid for protein synthesis, in 60% D₂O medium at 25°C. The relative radioactivity of each precursor was 18.5 kBq/0.2 μ M/ml. The incubated cells were washed with ice-cold water. The incorporation of amino acid into cells was determined from the radioactivity of cells using a liquid scintillation counter (LSC). That of nucleic acids was determined from the radioactivity in the ethanol-insoluble fraction of cells.

The effect of D₂O on protein synthesis was investigated using ³⁵S-L-methionine (³⁵S-Met; 185 kBq/0.2 μ M/ml) in 60 or 100% D₂O medium. Labeled cells (2×10^8 cells) were homogenized with glass beads. The homogenate was centrifuged at 200 $\times$ g for 10 min and then at 9,000 $\times$ g for 30 min, and the supernatant was applied to a Sephadex G-25 column (PD-10; Pharmacia Biotech). The radioactivity and protein concentration of these fractions were measured using LSC and a protein assay kit (Bio-Rad), respectively.

Carbon fixation and amount of photosynthetic pigments. Fixation of ¹⁴C was measured as described previously (13). Briefly, *Chlorella* cells (2×10^7 cells/190 μ l) were preilluminated with 2.5 klx for 3 min at 25°C, and then 10 μ l of 10 mM NaH¹⁴CO₃ (1.85 kBq/ μ l; Amersham) was added. The reaction was terminated by the addition of 0.8 ml of methanol. The mixture was added to 0.6 ml of 20% (vol/vol) acetic acid to remove unreacted NaH¹⁴CO₃, and cells were collected on a glass filter. The filter was washed with ice-cold water, and the radioactivity was counted in LSC.

Photosynthetic pigments were measured by collecting cells (6×10^8 cells) on a glass filter, grinding the filter in a mortar, then extracting pigments with 5 ml of 90% acetone. The concentrations of chlorophyll a and b and carotenoid were calculated from the absorption spectra as described previously (17).

Detection of Hsps and measurement of temperature sensitivity in *Chlorella*. Hsp70 in *Chlorella* cells was detected with anti-Hsp70 antiserum as described previously (18). Hsp60 was detected with the anti-Hsp60 monoclonal antibody against Hsp60 of *Yersinia enterocolitica* (Wako Pure Chemical Co.). The levels of Hsp60 and Hsp70 in the *Chlorella* cell lines were quantified with a densitometer (Shimadzu CS-9000).

To investigate the temperature sensitivity of these cells, the *Chlorella* cell lines were heat treated at 43°C for 1 h and then cultured at 25°C in H₂O medium for 3 days. Growth rates were estimated by counting cell numbers with a hemocytometer.

RESULTS

Selection of D₂O-resistant or -sensitive *Chlorella* mutants. Forty-eight *Chlorella* clones for which mutagenesis was induced with MNNG were analyzed for growth rate changes in medium prepared with 99.9% D₂O. We identified one clone as D₂O resistant among MNNG survivors. The clone known as DR-17 grew rapidly in D₂O medium, while wt cells were in lag

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FIG. 1. Growth rate of mutants of *Chlorella* cells in H_2O and D_2O medium. *Chlorella* cells (2×10^6 cells/ml) of wild type (wt, open circle), D-resistant mutant (DR-17, closed circle), and D-sensitive mutant (DS-24, closed square) were cultured in medium prepared with 99.9% D_2O or H_2O at $25^\circ C$ under 18 klx light and aerated with dry air.

phase (Fig. 1). Forty-eight *Chlorella* clones treated with EMS were also cultured in D_2O . EMS survivors did not produce any clones with a growth rate faster than wt cells in D_2O . Two clones had a growth rate slower than that of wt cells. One of these D_2O -sensitive clones was called DS-24. After a long lag phase, both wt cells and D_2O -sensitive clones could grow in D_2O medium. The most important difference between DR-17, wt, and DS-24 cell lines was the lag phase in the early stage of cultivation. The length of the lag phase of these cell lines was measured using synchronized cells. The lag time for DR-17 cells was 3.8 ± 1.1 days, while the lag times for wt and DS-24 were significantly longer at 5.7 ± 0.7 days and 7.4 ± 0.3 days, respectively. There was no difference in growth rate between clones and wt cells in H_2O medium (Fig. 1).

Characteristics of mutant clones. As the generation time of wt in 60% D_2O was about 48 h (17), the cells were incubated with thymidine for 48 h, with uridine for 4 h, or with α -AIB for 1 h. When the incorporation ratios of wt cells grown in 60% D_2O were compared to those in H_2O , those of thymidine, uridine, and amino acid were 0.81, 0.72, and 0.67, respectively. The protein synthesis measured with ^{35}S -Met was significantly suppressed to 0.25 in 60% D_2O and to 0.05 in 100% D_2O . These results show that protein synthesis was more significantly suppressed than DNA and RNA synthesis in D_2O . The rate of protein synthesis between wt and mutant cells cultured in D_2O medium was then compared. For adaptation to D_2O , wt and mutant cells were previously cultured in D_2O medium for 3 days and then cells were incubated in 100% D_2O medium for 1 h in the presence of ^{35}S -Met. The incorporation of ^{35}S -Met was higher in DR-17 cells than in wt or DS-24 cells grown in D_2O (Fig. 2).

The rate of carbon fixation of cells grown in the presence of D_2O was reduced to half the rate observed for both wt and the mutant clones not exposed to D_2O . The carbon fixation rate of

DR-17 cells was similar to that observed for wt cells grown in the presence of D_2O (Fig. 3). The carbon fixation rate tended to be lower in DS-24 cells than in wt and DR-17 cells. The level of the photosynthetic pigment, chlorophyll a, was slightly lower in DS-24 cells than in wt and DR-17 cells (data not shown) and may explain the lower carbon fixation rate observed in DS-24 cells.

Stress response in *Chlorella* cells against cultivation in D_2O and heat treatment. Protein synthesis in the cells is suppressed

FIG. 2. Protein synthesis of *Chlorella* mutants cultured in D_2O . wt, DR-17, and DS-24 *Chlorella* cells (2×10^6 cells/ml) were cultured in 100% D_2O medium for 3 days and then incubated in 100% D_2O medium for 1 h at $25^\circ C$ in the presence of ^{35}S -Met (185 kBq/0.2 μM /ml). Each value represents the mean $\pm$ standard deviation ($n = 3$; *, $P < 0.05$).

FIG. 3. Carbon fixation of *Chlorella* mutant cells in H₂O and D₂O. wt, DR-17, and DS-24 *Chlorella* cells (2×10^6 cells/ml) were illuminated in H₂O and 100% D₂O medium in the presence of NaH¹⁴CO₃ (18.5 kBq/10 μ l). Each value represents the mean $\pm$ standard deviation ($n = 3$).

under unfavorable conditions, such as higher temperature leading to the induction of heat shock proteins (Hsp, stress protein, molecular chaperone) (3, 11). To investigate the underlying mechanism of D resistance in DR-17 cells, the levels of Hsp in wt and mutant cells cultured in H₂O or D₂O medium for 3 days was compared. The level of Hsp60 was significantly higher in DR-17 cells compared to wt and DS-24 cells cultured in the presence of D₂O (Table 1). The level of Hsp70 was unchanged in these cells (data not shown). DR-17 cells were more resistant to heat treatment than wt and DS-24, when the resistance was estimated from the growth rate (Table 2).

DISCUSSION

The deuteration of recombinant proteins expressed in *Escherichia coli* and the methylotrophic yeast *Pichia pastoris* has been reported (4, 5, 8, 9, 12, 14). They can grow in high concentration of D₂O, and their components are labeled with D in exchangeable sites. While the growth rate of algae is slower than yeast or *E. coli*, algae cells have the advantage of photosynthetically producing D-enriched molecules from CO₂ with nonexchangeable deuterated sites. When various heterotrophs

TABLE 1. Relative level of Hsp60 in *Chlorella* mutant cells^a

Cell line	Relative level of Hsp60 with:	
	H ₂ O	D ₂ O
wt	1.00	1.62 $\pm$ 0.13
DR-17	1.66 $\pm$ 0.15	5.54 $\pm$ 0.47*
DS-24	1.15 $\pm$ 0.09	1.15 $\pm$ 0.08

^a Each value represents the mean $\pm$ standard deviation ($n = 3$; *, $P < 0.05$).

TABLE 2. Heat resistance of *Chlorella* mutant cells^a

Cell line	Relative growth rate
wt	1.00 $\pm$ 0.13
DR-17	1.60 $\pm$ 0.15*
DS-24	1.09 $\pm$ 0.10

^a wt, DR-17, and DS-24 *Chlorella* cells (2×10^6 cells/ml) were heat treated at 43°C for 1 h and then grown for 3 days in H₂O medium. Relative growth rates were generated from the cell number. Each value represents the mean $\pm$ standard deviation ($n = 3$; *, $P < 0.05$).

containing yeast and *E. coli* are cultured in H₂O using deuterated algae lysate, they can grow easily and the proteins expressed are labeled with D in nonexchangeable sites. These proteins will be useful for assignment in NMR analysis (6). Although a D-enriched algal cell lysate is commercially available, the growth of cells is significantly suppressed in the presence of high concentrations of D₂O. Development of the D-resistant *Chlorella* mutant from this investigation provides a rapid and effective source of D-enriched algal cell lysate and D-labeled biomolecules.

It is well known that prior cultivation of algae and microorganisms in a low concentration of D₂O aids the subsequent cultivation of cells in a high concentration of D₂O (1, 2, 10). Actually, prior cultivation in D₂O decreased the suppressive effect on protein synthesis and carbon fixation in wt and mutant cells (data not shown). This adaptation process is similar to the induction of heat resistance in that treatment of cells at a sublethal temperature induces Hsp (molecular chaperone), allowing cells to become more resistant to subsequent treatment at higher, more lethal temperatures. Molecular chaperones are thought to have protective and repairing roles providing protein stability and allowing accurate folding and assembly during stressful conditions. The growth rate of DR-17 in D₂O was higher than those of wt and DS-24. The enhanced induction of Hsp60 may explain the observed resistance of the *Chlorella* mutant to heat treatment and also explain the resistance of this mutant to the harmful solvent isotope effect of D₂O. Previously, we isolated a D-resistant yeast mutant with similar characteristics expressing increased levels of Hsp70 (15). The increased expression of Hsp in both D-resistant yeast and *Chlorella* suggests that D-resistant mutants of other autotrophic and heterotrophic cells may be produced.

In this study, we generated a D₂O-resistant mutant cell of algae *Chlorella* that grows more rapidly in the presence of D₂O than wt cells. The level of molecular chaperone, Hsp60, was higher in the mutant than in the wt cell line cultured in D₂O. This mutant provides a useful source of D-enriched nutrients for heterotrophic cells such as yeast and animal cells.

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