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Reports

Tetrahydropteridines possess antioxidant roles to guard against glucose-induced oxidative stress in *Dictyostelium discoideum*

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Glucose effects on the vegetative growth of *Dictyostelium discoideum* Ax2 were studied by examining oxidative stress and tetrahydropteridine synthesis in cells cultured with different concentrations (0.5X, 7.7 g L^{-1}; 1X, 15.4 g L^{-1}; 2X, 30.8 g L^{-1}) of glucose. The growth rate was optimal in 1X cells (cells grown in 1X glucose) but was impaired drastically in 2X cells, below the level of 0.5X cells. There were glucose-dependent increases in reactive oxygen species (ROS) levels and mitochondrial dysfunction in parallel with the mRNA copy numbers of the enzymes catalyzing tetrahydropteridine synthesis and regeneration. On the other hand, both the specific activities of the enzymes and tetrahydropteridine levels in 2X cells were lower than those in 1X cells, but were higher than those in 0.5X cells. Given the antioxidant function of tetrahydropteridines and both the beneficial and harmful effects of ROS, the results suggest glucose-induced oxidative stress in *Dictyostelium*, a process that might originate from aerobic glycolysis, as well as a protective role of tetrahydropteridines against this stress. [BMB Reports 2013; 46(2): 86-91]

INTRODUCTION

The multicellular slime mould *Dictyostelium discoideum* Ax2 is notorious for its production of two isomeric forms of tetrahydropteridines, D-threo-H4-biopterin (DH4) and L-erythro-tetrahydrobiopterin (BH4) (1). BH4 is a well-known cofactor of aromatic amino acid hydroxylases and nitric oxide synthases (NOSs) in mammals (2). BH4 also functions as an antioxidant, directly scavenging reactive oxygen species (ROS), and as a regulator of NOSs (2, 3). Under oxidative stress conditions, levels of BH4 are diminished, causing NOS uncoupling. The uncoupled NOS produces superoxide anions instead of nitric oxide, further exacerbating oxidative stress, and contributing to mitochondrial dysfunction and eventual cellular demise. In *Dictyostelium*, DH4 is quantitatively dominant over BH4 and seems to be the preferred antioxidant, while BH4 functions as a cofactor of phenylalanine hydroxylase (4, 5). Because NOS homologs have not been identified in the predicted genome sequence of *Dictyostelium*, DH4 may be used as a direct radical scavenger in the organism. The enzymatic machinery for tetrahydropteridine synthesis in *Dictyostelium* consists of GTP cyclohydrolase I (GTPCH), 6-pyruvoyl tetrahydropterin synthase (PTPS), sepiapterin reductase (SR), and aldose reductase (AR) (6). Through their cofactor and antioxidant functions tetrahydropteridines are converted to dihydro forms, which are recycled back into tetrahydro forms by enzymes, including dihydropteridine reductase (DHPR) and/or dihydrofolate reductase (DHFR) (5). Because the tetrahydro forms are active *in vivo*, both the synthesis and regeneration pathways are essential for maintenance of the quantitative requirement and the functional integrity of tetrahydropteridines.

Although *Dictyostelium* feeds on bacteria in nature, glucose is required for vegetative growth in axenic media; significant growth was not observed in a minimal medium lacking glucose (7). Glucose is stimulatory to the growth of *Dictyostelium* in a complex medium (HL5), but becomes inhibitory above an optimal level (8). Although high levels of glucose are commonly known to induce oxidative stress in mammalian cells and yeast (9, 10), there has been no relevant study of such effects in *Dictyostelium*. Furthermore, while studying *Dictyostelium* cultures, we found that levels of tetrahydropteridine in vegetatively growing cells vary, depending on the glucose concentration in the HL5 medium. We assumed that the glucose-dependent change in tetrahydropteridine levels is closely associated with their antioxidant function, in which they detoxify ROS increases induced by glucose. In order to challenge the assumption, we examined oxidative stress and tetrahydropteridine production in cells cultured in HL5 medium which was supplemented with different concentrations of exogenous glucose. Here, we report our findings on the functional role of tetrahydropteridines in glucose-induced oxidative stress and the putative involvement of aerobic glycolysis in the vegetative growth of *Dictyostelium*.

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RESULTS

Cell growth and tetrahydropteridine production

The effects of glucose on vegetative growth and tetrahydropteridine synthesis in *Dictyostelium discoideum* Ax2 were examined with three different concentrations of glucose (0.5X, 7.7 g L^{-1}; 1X, 15.4 g L^{-1}; 2X, 30.8 g L^{-1}) in HL5 medium. Consistent with previous findings (8), the growth rate was optimal in 1X cells (cells grown in 0.5X glucose) but was drastically reduced in 2X cells, below the levels seen in 0.5X cells (Fig. 1A). The extent of cellular tetrahydropteridine production was determined by the amount of pteridines (the fully oxidized form of tetrahydropteridines) normalized by total protein content (Fig. 1B). Compared with 0.5X cells, 1X cells showed 5-fold higher levels of tetrahydropteridines. It was interesting that 2X cells also maintained at least a 3-fold greater level of tetrahydropteridines than 0.5X cells did, in spite of having a much slower growth rate.

Glucose-induced oxidative stress

To examine the growth-inhibitory effects of 2X glucose (Fig. 1A) we evaluated various markers of oxidative stress in the cells cultured with added glucose (Fig. 2). Flow cytometry results revealed that the number of ROS-positive cells was increased in a dose-dependent manner (Fig. 2A), supporting the theory of glu-

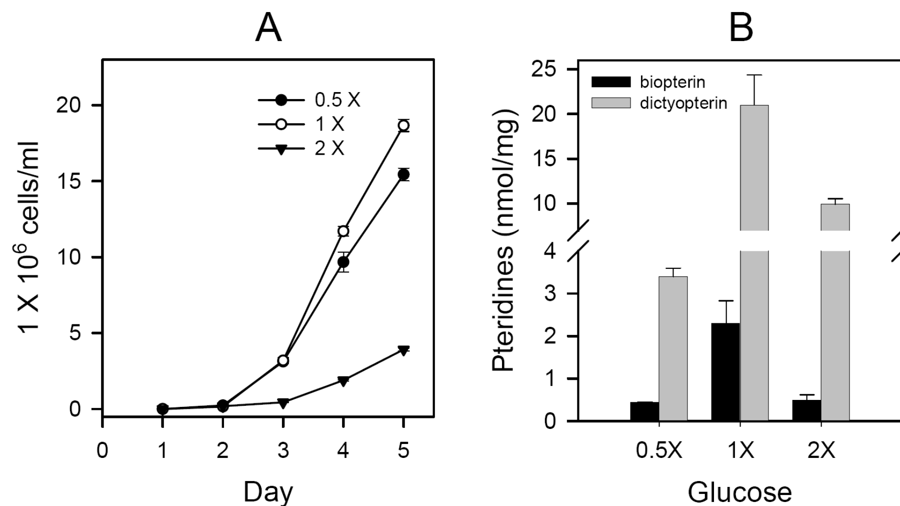

Fig. 1. Cell growth and intracellular pteridines. (A) Cells grown in HL5 medium containing the indicated amounts of glucose were counted using a hemacytometer. (B) Intracellular levels of BH4 and DH4 were determined by HPLC as oxidized forms, biopterin (dark) and dictyopterin (grey), respectively.

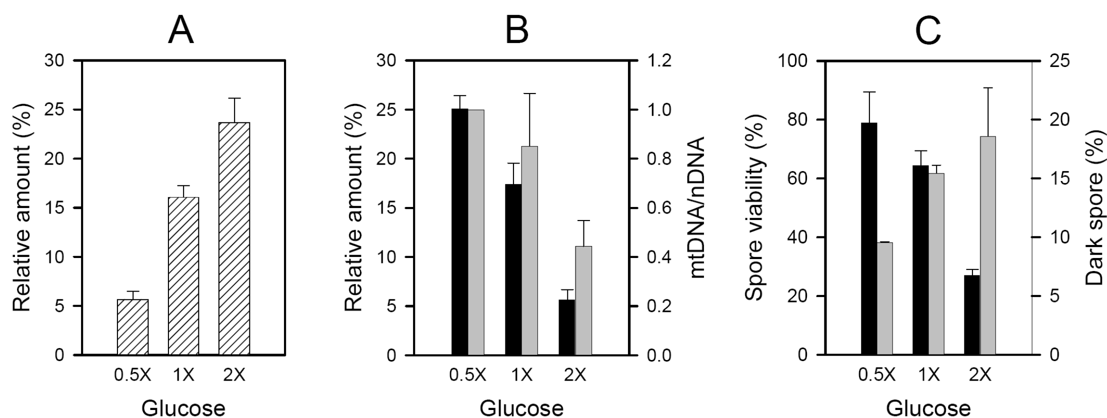

Fig. 2. Evaluation of oxidative stress. (A) ROS measured by DCFH-DA staining. The fluorescent cells were analysed at 525 nm using FACS. (B) Mitochondrial activity. Mitochondrial transmembrane potential measured by JC-1 staining (dark, left axis). The active red fluorescent cells were analysed at 485 nm using FACS. Quantitative real-time PCR of mtDNA and nDNA (grey, right axis). The mitochondrial COX-I gene was quantified relative to that of the nuclear GAPDH gene. (C) Spore viability was counted as plaques on an *E. coli* lawn (dark, left axis). Dark spores were counted using a phase contrast microscope (grey, right axis).

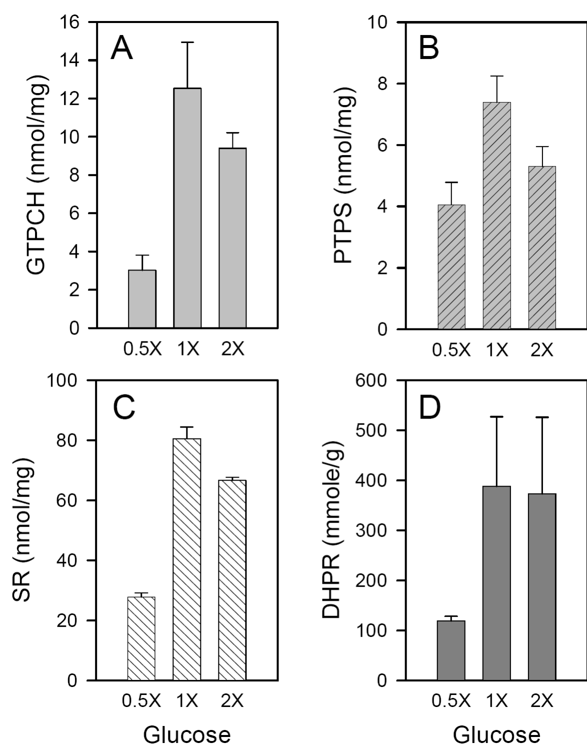

Fig. 3. Specific activities of the enzymes involved in the *de novo* synthesis and regeneration of tetrahydropteridines. (A) GTPCH activity measured as neopterin. (B) PTPS activity measured as pterin. (C) SR activity measured as the sum of biopterin and dictyopterin. (D) DHPR activity measured as oxidized NADH.

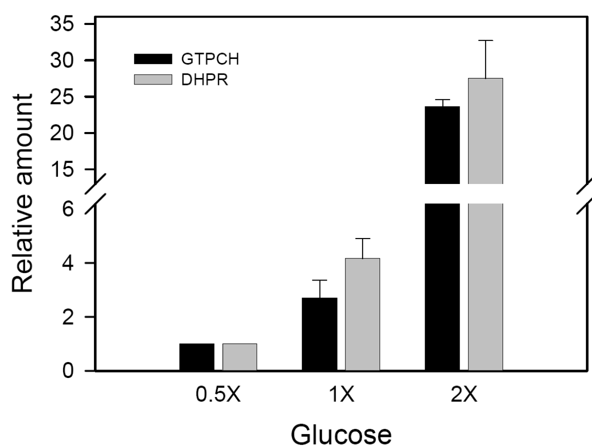

Fig. 4. Gene expression levels of GTPCH and DHPR. The mRNA copy numbers of GTPCH (dark) and DHPR (grey) were quantified relative to that of GAPDH by real-time PCR.

cose-induced accumulation of ROS. Mitochondrial function was also evaluated by counting the cells exhibiting increased transmembrane potentials, and by quantifying mtDNA copy numbers. Both values were reduced by exogenous glucose (Fig. 2B). Spore viability was also reduced progressively (Fig. 2C), along with a concomitant increase in the number of phase-dark spores (Fig. 2C). Because phase-dark spores are known to originate from mitochondria with disrupted cristae (11), it seemed that irreversibly damaged mitochondria deteriorated the viability of spores, which were known to depend more on aerobic respiration (12). Taken together, the results strongly support the glucose-dependent increase of oxidative stress and mitochondrial dysfunction in *Dictyostelium* cells, thereby suggesting that glucose-induced oxidative stress may be responsible for the poor cell growth observed at the high glucose concentration (2X).

Regulation of tetrahydropteridine production

To further examine the effects of glucose on tetrahydropteridine synthesis, we first determined the specific activities of the enzymes involved in synthesis and regeneration (Fig. 3). All enzymes were highly similar to each other in their quantitative changes in specific activity with varying glucose levels: The enzyme activities were lower in 2X cells than in 1X cells, except in terms of DHPR activity, which was not much different between them, but enzyme activities were still higher in 2x cells than in 0.5X cells. Compared to 0.5X cells, 1X cells exhibited a greater than 4-fold increase of GTPCH activity, which is the rate-limiting enzyme for tetrahydropteridine synthesis, to support the increased tetrahydropteridine production (Fig. 1B). DHPR, a crucial enzyme involved in the recycling of tetrahydropteridines, was also approximately 4 times more active in 1X cells than in 0.5 X cells.

In order to see if the enhanced specific activities represented a transcriptional activation of the encoding genes, the expression levels for the genes encoding GTPCH and DHPR were determined by real-time PCR (Fig. 4). Remarkably, the ratios of the copy numbers between the 0.5X and 1X cells were in good agreement with those of their enzyme activities. In contrast, the mRNA copy numbers in 2X cells were more than 20-fold higher than those in 0.5X cells, inconsistent with enzyme activities and tetrahydropteridine levels. These apparently inconsistent results exhibited by 2X cells, however, might have resulted from overproduction of ROS which exceeded a toxic threshold level, given the glucose-induced ROS increase (Fig. 2A) as well as both the beneficial and destructive actions of ROS (13, 14), as discussed below.

DISCUSSION

In this study, it was determined that cell growth and tetrahydropteridine synthesis of *Dictyostelium discoideum* were enhanced at an optimal level of glucose (1X) but were impaired at a higher level (2X), while ROS production was increased consistently with glucose. The results may suggest a close association between cell growth and tetrahydropteridine production under conditions of oxidative stress induced by ROS. Although some of the results appear contradictory to others, they may be

interpreted in terms of the antioxidant function of tetrahydropteridines (4) and the janus-faced nature of ROS (13). The antioxidant function of tetrahydropteridines in *Dictyostelium* was already demonstrated via targeted disruption of the gene encoding SR or DHPR (4, 5). The mutants impaired in tetrahydropteridine production were poor in vegetative growth, mitochondrial function, and spore viability and were more susceptible to oxidative stress than the wild type. It is also widely accepted that a moderate level of ROS may be beneficial to cell proliferation, whereas excessive ROS may overwhelm the cellular antioxidant capacity and trigger cell death (13, 14). In our results, 1X cells showed better growth than 0.5X cells (Fig. 1A), in spite of producing more ROS (Fig. 2B). The higher levels of tetrahydropteridines in 1X cells (Fig. 1B), which agree well with the enhanced enzyme activities (Fig. 3) and gene expressions (Fig. 4), may have contributed to the optimal growth by counterbalancing the ROS increase. On the other hand, 0.5X cells, which were under relatively low oxidative stress conditions (Fig. 2), produced lesser amounts of tetrahydropteridines but were able to grow well. Further increasing ROS level in 2X cells would have prevailed over tetrahydropteridine production to suppress cell growth. Although excess ROS may be still stimulatory to the gene expression (Fig. 4), it may be also harmful to the corresponding enzymes (Fig. 3) and tetrahydropteridines (Fig. 1B). Under oxidative stress conditions proteins are oxidatively modified by ROS and subsequently degraded by the proteasome, which is the primary intracellular proteolytic pathway implicated in the degradation of oxidized proteins (15). As *Dictyostelium* also features a 20S proteasome (16), we determined the activity in the cells cultured with glucose (Supplementary data Fig. S1). The proteasome activity was increased with higher glucose levels. Although an indirect evidence, it may support the reduced enzyme activities in 2X cells (Fig. 3). It is also well-known that tetrahydropteridines are prone to oxidation and subsequent degradation by ROS (3), supporting the observation that the tetrahydropteridine production levels in 2X cells were half those of the 1X cells (Fig. 1B), although the enzyme activities did not differ significantly (Fig. 3). In conclusion, we suggest that tetrahydropteridines play a role of neutralizing the harmful effect of ROS via their antioxidant function during vegetative growth in glucose medium. If the quantitative balance between tetrahydropteridines and ROS is impaired by overproduction of ROS in high glucose conditions, cells succumb to oxidative stress.

We have demonstrated for the first time the glucose-dependent increase in ROS and mitochondrial dysfunction in vegetative cells of *Dictyostelium* (Fig. 2). We are curious whether a metabolic switch from mitochondrial respiration to fermentation occurs in the cells cultured in high glucose. The metabolic switch to aerobic glycolysis is known in many unicellular and multicellular organisms, including yeasts and human cancer cells (17, 18). Consistent with the result (Fig. 2), the yeast *Saccharomyces cerevisiae* releases fewer ROS from mitochondria in conditions of low glucose (19). *Dictyostelium* was also known to grow in the same manner as a facultative anaerobe, although the morphogenetic sequence is obligately aerobic (16). It was suggested that this kind of metabolic switch is a trade-off between yield and growth rate in heterotrophic organisms, so that the enhanced aerobic glycolysis can allow high-rate ATP production with a selective advantage when competing for limited resources (17). We are not sure, however, if *Dictyostelium* competes with bacteria for glucose in natural habitats, where *Dictyostelium* feeds on bacteria, which are nonfermentable resources. Additionally, aerobic glycolysis in cancer cells is considered to be a means of minimising oxidative stress by diverting substrates from the mitochondrial electron transport chain (20), which is a major source of intracellular ROS (21). For *Dictyostelium*, the putative aerobic glycolysis pathway, which has yet to be proven, could be a means for the cells to avoid ROS burst in a high-glucose axenic medium.

MATERIALS AND METHODS

Cells and growth conditions

Axenic cultures of Ax2 cells were grown at 22°C in a rotary shaker. Three different concentrations of glucose (0.5X, 7.7 g/L; 1X, 15.4 g/L; 2X, 30.8 g/L) were used in HL5 medium (7.15 g yeast extract, 14.3 g protease peptone, 0.485 g KH_2PO_4, 1.28 g $Na_2HPO_4 \cdot 12H_2O$, pH 6.5, per litre) with 100 μg/ml streptomycin sulphate and 100 U/ml benzylpenicillin potassium (22).

Spore function assay

Spore integrity was examined via a viability assay and phase-dark spore counting as described previously (4, 23). Cells (2×10^6 cells/ml) were harvested by centrifugation at 350 × g for 3 min, resuspended in 12 mM cold phosphate buffer, pH 6.5 (PB), and were washed three times. Finally, the cell suspension was adjusted to a density of 1×10^8 cells/ml and was deposited on a Whatman #50 filter paper that was placed on a non-nutrient agar plate containing PB, and left for 24 h. Two days after the appearance of fruiting bodies, spores were harvested, by banging the inverted plates on the bench, and were collected with cold PB. The spore suspension was adjusted to 1×10^4 cells/ml and was incubated at 37°C for 30 min to germinate. An aliquot containing 100 spores was spread on a nutrient agar plate in association with *Escherichia coli*. After incubation for 3 days at 22°C, plaques were counted as viable spores. Before plating with *E. coli*, an aliquot of the spore suspension was also counted for ovoid phase-dark spores with a hemocytometer.

Analyses of ROS production and mitochondrial transmembrane potential

Cells stained with specific dyes were counted, employing a FACS machine as described previously (4). One ml of exponentially growing cells (2×10^6 cells/ml) was washed twice with low fluorescence axenic medium (11 g glucose, 5 g casein peptone, 5 mM K_2HPO_4, 0.5 mM NH_4Cl, 0.2 mM $MgCl_2$, 0.01 mM $CaCl_2$, 0.05 mM $FeCl_3$, 0.484 mg Na_2-EDTA $\cdot$ $2H_2O$, 0.23 mg $ZnSO_4$, 0.111 g H_3BO_4, 0.051 mg $MnCl_2 \cdot 4H_2O$, 0.017 mg $CoCl_2 \cdot 6H_2O$, 0.015

mg $CuSO_4 \cdot 5H_2O$, 0.01 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) and was incubated in the same medium for 30 min. Cells were stained with 200 nM JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide) dissolved in the same medium for 30 min at 22°C in a dark room. After being washed in the same medium, 1×10^4 cells in fresh PB were analysed by FACSCaliber (BD Bioscience) at 485 nm.

DCFH-DA (dichlorofluorescein diacetate) staining followed the procedure of JC-1 staining, except that cells were incubated in 100 nM DCFH-DA for 45 min. The deacetylated DCFH is preferentially oxidized by H_2O_2 and superoxide to the fluorescent compound dichlorofluorescein (DCF), which was analysed at 525 nm.

Quantitative real-time PCR

Dictyostelium total RNA was extracted from vegetative cells using TRI reagent (Molecular Research Center Inc.). cDNA was prepared from 2 μg of total RNA using the QIAGEN One Step RT-PCR Kit (QIAGEN). The partial ORF sequences of GTPCH, DHPR, and GAPDH were quantified separately with Rotor-Gene 3000 (Corbett Life Sci., Australia). The RT-PCR reactions were carried out using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the following primer pairs: GTPCH upper, 5′-CATCATATGGTGCCATTCCA; GTPCH lower, TGAGCTTGAATTGCTTGTGC, DHPR upper, 5′-GGTTGGTCA GGTGGTAATGC, DHPR lower, 5′-ATTCAATGCTGCTGATGCA C, GAPDH upper, 5′-GGTCGTATCGGTCGTCTTGT, and GAP DH lower, 5′-GCGAAAACGTGGATTTTGTT. A typical 10 μl reaction mixture contained 5 μl Master Mix, 0.2 pmol primers, and template DNA. An initial denaturation step (2 min at 95°C) was followed by 40 cycles (20 s at 94°C, 20 s at 53°C, 20 s at 72°C).

The ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) was also determined by RT-PCR. Total DNA was isolated from exponentially growing cells using the LaboPass Genomic DNA isolation Kit (Cosmo Co. Ltd., Korea). The nuclear gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the mitochondrial gene cytochrome c oxidase subunit I (COX-I) were quantified separately by PCR, as described above, using specific primers: GAPDH upper, 5′-TCA ACTGATGCCCCAATGTA; GAPDH lower, 5′-CGTGAACGGTG GTCATTAAA; COX-I upper, 5′-ACAAACTAAATGCGGGAACG; COX-I lower, 5′-TTAAATTTACGCCCCCACAG. An initial denaturation step (10 min at 95°C) was followed by 40 cycles (30 s at 95°C, 30 s at 53°C, 20 s at 72°C).

Enzyme assays

The activities of GTPCH, PTPS, and SR were determined from the cell lysates following the methods described previously (24). Exponentially growing cells (2×10^6 cells/ml) were harvested and dissolved in 10 mM Tris–HCl (pH 7.0), 1 mM DTT, and 1 mM PMSF. The cells were disrupted by three cycles of freeze-thawing, and were centrifuged to remove precipitates.

GTPCH assay was performed in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM EDTA, 4 mM DTT, and 1 mM GTP. The reaction was terminated by adding an equal volume of acidic iodine solution (KI/I_2, 2%/1%, in 1 N HCl) and the disrupted cells were then left in the dark for 1 h. After centrifugation, the supernatant was mixed with ascorbic acid and NaOH, after which it was incubated with alkaline phosphatase for dephosphorylation of neopterin triphosphate.

PTPS assay was performed in a reaction mixture of 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 5 mM N-acetyl-5-hydroxytryptamine, and an aliquot of a dihydroneopterin triphosphate mixture, which was prepared from GTP by incubation with recombinant *Synechocystis* sp. PCC 6803 GTPCH. The reaction was stopped by adding a double volume of acidic iodine solution.

SR activity was assayed in the reaction mixture of 50 mM potassium phosphate (pH 6.5), 1 mM sepiapterin, and 0.2 mM NADPH, followed by mixing with an equal volume of acidic iodine solution. The mixtures were left for 0.5-1 h at room temperature in the dark, centrifuged to remove protein precipitate, and were then mixed with 5% ascorbic acid to reduce excessive levels of iodine. Oxidized pteridines were analysed using HPLC equipped with an Inertsil ODS-3 (5 μm, 150 × 2.3 mm, GL Science, Japan) and a fluorescence detector (Shimadzu RF-10AXL). Pteridines were eluted with 10 mM sodium phosphate (pH 6.0) at a flow rate of 1.2 ml min^{-1} and monitored at 350 nm/450 nm (ex/em). Authentic pteridines were purchased from Dr. Schircks Laboratories (Jona, Switzerland).

DHPR activity was measured by monitoring the oxidation of NADH via the reduction of the quinonoid isomer to tetrahydropterin (4). The assay mixture contained 50 mM Tris-HCl (pH 7.5), 0.004% H_2O_2, 0.15 mM NADH, 5 units peroxidase, and 1 mM BH4 in a final volume of 630 μl. The reaction was initiated by adding the substrate, and the mixture was then incubated for 5 min at 25°C. The absorbance of the samples and blanks (which lacked enzyme) was measured at 340 nm. Subsequently, 70 μl of crude extract was added, and the absorbance was measured at the same wavelength. The consumption of NADH was measured against corresponding blanks at 340 nm for 5 min.

Proteasome activity assay

The 20S proteasome function was measured as described previously (25). Briefly, exponentially growing cells (2×10^6 cells/ml) were washed with PBS and then with lysis buffer (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP). The cells were sonicated briefly and were then centrifuged at 10,000 ×g for 20 min. The crude extract (100 μg protein) was diluted with the lysis buffer to a final volume of 1 ml, and was incubated with 80 μM fluorogenic substrate Suc-LLVY-7-amido-4-methylcoumarin dissolved in 1% DMSO. The activity to produce free 7-amido-4-methylcoumarin was measured with a fluorescence plate reader (GENios Plus, Tecan) at 360/450 nm.

Statistical analysis

All of the experiments described above were performed in triplicate or more, and the results were expressed as the mean ± S.D.

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