Visualization of the microbody division in *Cyanidioschyzon merolae* with the fluorochrome brilliant sulfoflavin

Rapid communication

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Summary. A novel procedure is described for fluorescence staining of microbodies, which can be applied quickly and easily. We developed this technique of microbody staining with the unicellular red alga *Cyanidioschyzon merolae*. *Cyanidioschyzon merolae* only contains a single chloroplast, mitochondrion, and microbody per cell, and the mitotic cycle and the organelle division cycle are easily synchronized. Knowing that the concentration of H$_2$O$_2$ in the microbody is higher than it is in the cytosol and other cell components, we attempted to visualize the microbody by using fluorescence microscopy to detect H$_2$O$_2$. Brilliant sulfoflavin (BSF), used for detecting Fe$^{2+}$ in analytical chemistry, fluoresces when it reacts with Fe$^{2+}$ and H$_2$O$_2$. We were able to specifically stain microbodies with BSF, under acidic conditions (pH 3.0 or pH 2.5) with blue-light excitation. Using this procedure, we observed division of the microbody and the effect of aphidicolin on the microbody. We also discovered that microbody division is regulated by the cell nucleus and follows division of the cell nucleus.

Keywords: Brilliant sulfoflavin; *Cyanidioschyzon merolae*; Fenton reaction; Fluorescence microscopy; Hydrogen peroxide; Microbody.

Introduction

Microbodies (peroxisomes, glyoxysomes, and related organelles) are essential subcellular organelles found in virtually all eukaryotic cells (Frederick et al. 1968, Hruban et al. 1972). They are typically spherical or spheroidal in shape, between 0.2 and 1.0 µm in diameter and surrounded by a single membrane.

Until now, microbodies were stained for transmission electron microscopy (TEM) either by the alkaline 3,3’-diaminobenzidine (DAB) method, which detects catalase activity cytochemically (Fahimi 1968, 1969), or by labeling catalase or other microbody marker proteins with protein A-gold or immuno-gold labeling (Baumgart et al. 1989). The DAB method and protein A-gold technique have also been used to detect proteins in thin sections with light microscopy; the proteins appear as black particles (Litwin et al. 1984, Beier and Fahimi 1987, Beier 1992). However, these methods require several treatments and are time-consuming. It should be possible to use indirect immunofluorescence to detect marker proteins and directly visualize microbodies by light microscopy (Baumgart 1994). Visualization by light microscopy would make it possible to observe the entire structure of large numbers of microbodies at once.

In this study, we developed a new technique for visualizing microbodies by light microscopy, using microbody-specific fluorescence staining with brilliant sulfoflavin (BSF). This technique detects H$_2$O$_2$ in microbodies. Many oxidative and metabolic reactions, which occur in microbodies, produce hydrogen peroxide (H$_2$O$_2$) as a by-product. Catalase decomposes this H$_2$O$_2$, but since the $K_m$ of catalase is comparatively high, 2.5 × 10$^{-2}$ M, the concentration of H$_2$O$_2$ in the microbodies is considerably higher than in the cytosol and other organelles. We searched for a flu-
orescent dye that detects H$_2$O$_2$. Brilliant sulfoflavin (BSF, sodium 4-amino-N-(p-tolyl)-naphthalimide-3-sulfonate, C$_{19}$H$_{13}$N$_2$O$_4$SNa) reacts with Fe$_{2+}$ and H$_2$O$_2$ in the Fenton reaction and emits chemiluminescence (Yamada et al. 1985, Elrod et al. 1991),

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot\text{OH}.$$ 

We chose to test this procedure for staining microbodies in the unicellular red alga *Cyanidioschyzon merolae*. There are two advantages observing microbodies in *C. merolae*. It contains only a single chloroplast, mitochondrion, and microbody per cell, and the mitotic cycle and the organelle division cycle can be synchronized on a 12-h light/12-h dark regime (Suzuki et al. 1994). We were able to visualize microbodies by light microscopy using the specific fluorescence of BSF and also observed microbodies dividing.

**Material and methods**

**Cell culture**

*Cyanidioschyzon merolae* cells were cultured in Allen’s (1959) medium at pH 2.5, in L-type culture tubes or flasks with shaking, under continuous light (40 W/m$^2$) at 40 °C. The cells were then synchronized on a 12-h light/12-h dark regime at 40 °C while the medium was aerated with 5% CO$_2$. For aphidicolin treatments, 10 μg of aphidicolin per ml were added to the medium at the start of the second dark period, and the cells were then cultured in continuous darkness.

**Fluorescence microscopy with BSF staining**

*Cyanidioschyzon merolae* cells were harvested in the second dark period by centrifugation at 2000 g for 5 min. After the supernatant was removed, the cells were fixed in 1% glutaraldehyde dissolved in medium. Then the cells were washed twice with one of a number of buffers. These buffers included: Allen’s medium, 100 mM citric acid-sodium citrate buffer (citrate buffer) at pH 3.0, pH 4.0, and pH 6.0, 100 mM Tris-HCl at pH 7.6, 100 mM citrate buffer at pH 3.0 with either 500 mM Fe$^{2+}$ (FeSO$_4$) or 1 mM Fe$^{3+}$, and citrate buffer at pH 6.0 with either 500 mM Fe$^{2+}$ or 1 mM Fe$^{3+}$. The cell suspension was stained with BSF at a concentration of 30 μg/ml. The BSF fluorescence was observed under blue-light excitation. Since chloroplasts contain chlorophyll a, chloroplasts were observed emitting a red autofluorescence when they were excited with green light. The samples were examined under a BHS-RFC epifluorescence microscope (Olympus, Tokyo, Japan).

**Electron microscopy**

The cells were collected by centrifugation and prefixed in 1% glutaraldehyde dissolved in medium for 10 min. The prefixed cells were dissolved in 20 mM sodium cacodylate (pH 7.2) plus 30% glycerol and immediately collected by centrifugation. The cells were then rapidly frozen in liquid propane that had been cooled with liquid nitrogen (−195 °C). The frozen cells were transferred to 1% OsO$_4$ dissolved in dry acetone at −80 °C, and incubated for 48 h. Subsequently, the samples were warmed gradually by holding them for 2 h at −20 °C, then 2 h at 0 °C, and finally for 1 h at room temperature. The samples were washed with dry acetone at room temperature and embedded in either Spurr’s resin or LR White. The thin sections (90 nm thick) were stained with uranyl acetate and lead citrate, and examined with a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

**Results**

**Determination of conditions for BSF staining**

First, we determined the optimal conditions for BSF staining. BSF reacts with H$_2$O$_2$ and Fe$^{2+}$ (Fenton reaction) and emits fluorescence. Cells were fixed with 1% glutaraldehyde and suspended in a variety of buffers with 30 μg of BSF per ml, in order to determine the optimal pH and whether exogenous Fe$^{2+}$ was required for specific fluorescence (Table 1). No fluorescence was detected without exciting light under any condition. With blue-light excitation, no site-specific fluorescence was observed under neutral conditions (Table 1 and Fig. 1 A, B). While 1 mM exogenous Fe$^{2+}$ had no effect, the addition of 500 mM Fe$^{2+}$ produced a small amount of site-specific fluorescence. However, under acidic conditions (Allen’s medium and citrate buffer at pH 3.0) a round structure about 0.3 μm in diameter, which specifically fluoresced, was observed above the chloroplast, beside the mitochondrion. The nucleus, mitochondrion and chloroplast all emitted small amounts of fluorescence (Table 1 and Fig. 1 C, D). The addition of either 1 mM or 500 mM Fe$^{2+}$ had no discernible effect (Table 1).

To confirm that the round structure stained by BSF

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>7.6</td>
<td>–</td>
</tr>
<tr>
<td>Citrate buffer</td>
<td>6.0</td>
<td>–</td>
</tr>
<tr>
<td>Citrate buffer</td>
<td>4.0</td>
<td>+</td>
</tr>
<tr>
<td>Citrate buffer</td>
<td>3.0</td>
<td>+++</td>
</tr>
<tr>
<td>Allen’s medium</td>
<td>2.5</td>
<td>+++</td>
</tr>
<tr>
<td>Citrate buffer</td>
<td>6.0</td>
<td>–</td>
</tr>
<tr>
<td>with 1 mM Fe$^{3+}$</td>
<td>6.0</td>
<td>+</td>
</tr>
<tr>
<td>with 500 mM Fe$^{2+}$</td>
<td>3.0</td>
<td>+++</td>
</tr>
<tr>
<td>with 500 mM Fe$^{2+}$</td>
<td>3.0</td>
<td>+++</td>
</tr>
</tbody>
</table>

Results are scored in arbitrary units from – to +++