Sol–Gel Encapsulated Light-Transducing Protein Phycoerythrin: A New Biomaterial

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An optically transparent biomaterial is produced by encapsulating a light-transducing protein, phycoerythrin, in a sol–gel matrix. Absorption and fluorescence measurements indicate that the protein not only retains its native optical properties in the sol–gel matrix but also shows an enhanced stability toward photodegradation. Two-photon induced fluorescence is observed from phycoerythrin in both solution and sol–gel matrices. The potential application of this biomaterial in biosensors, three-dimensional biomolecular imaging, and three-dimensional optical storage is discussed.

Introduction

Materials based on biological macromolecules have received considerable attention for potential application in optical information processing and biosensor devices.[1–5] Examples are the light-harvesting light-transducing proteins, such as bacteriorhodopsin (bR) and phycobiliproteins. Although photoactive biological proteins have been extensively investigated for their light-transducing properties, only recently has the possibility of using such proteins as active components of photonic devices been explored. Bacteriorhodopsin has already demonstrated great promise as a potential optical material in dynamic holograms for pattern recognition,[3] artificial photoreceptors for image processing,[2,5,6] bipolar artificial neurons for neural networks,[7] and storage medium for 3-D optical memory.[1,8] Phycobiliproteins represent another important class of light-transducing proteins that may support applications similar to those already demonstrated with bacteriorhodopsin.[9]

Phycobiliproteins are biomolecular assemblies located on the outer thylakoid membranes of marine algae. They are light-transducing proteins that harvest and funnel ambient light into the photoreactive center to drive photosynthesis.[10] The phycobiliproteins (phycoerythrin, phycocyanin, and allophycocyanin) are organized as stacked disks in the phycobilisome, each with a region of maximum and relatively narrow wavelength of absorption in the visible spectrum. In this unique arrangement phycobiliproteins are able to efficiently absorb and transfer light energy at low intensity levels via a Forster mechanism to chlorophyll for photosynthesis with greater than 90% efficiency.[11]

The individual phycobiliproteins possess very interesting optical properties and are widely used today as fluorescent markers in biochemical and biomedical research.[12] We will focus on the outermost phycobiliprotein from the thylakoid membrane, phycoerythrin (PE). Phycoerythrin is a bulky, water-soluble protein that displays very intense fluorescence (more than 20 times larger than fluorescein) with high quantum yields and a large Stokes shift (a factor of 2.7 larger than fluorescein).[12] In dried thin-film form, phycoerythrin has demonstrated the ability to retain these optical properties as well as exhibit some interesting photovoltaic and photoconducting behavior.[13] These interesting optical and optoelectronic properties make phycoerythrin a promising candidate for signal transduction and biosensor applications.

However, to demonstrate the feasibility of phycobiliproteins for optical devices, PE-based biomaterials with high optical quality and good thermal and mechanical stability first have to be developed. A number of approaches have been attempted to manipulate these biomaterials into useful architectures while still maintaining the desired optoelectronic properties of the protein. These include both the application of the Langmuir–Blodgett (LB) technique and the incorpora-

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tion of protein into water-soluble polymers such as poly(vinyl alcohol).[4,9,14] In our previous studies, we have designed a generic "cassette" system that can incorporate biotinylated phycobiliprotein into a conducting polymer LB film matrix using the streptavidin–biotin interaction.[4] This system may be applied to any host of biomolecules that can be biotinylated. However, many applications require thick films with high optical quality and good thermal and mechanical stability. Recently, several groups have reported the incorporation of biological molecules into inorganic transparent glass using the sol-gel technique.[15–17] The encapsulation of bR in a transparent sol–gel glass has also been reported by Wu et al. and Weetall et al.[18,19] The sol–gel technique involves the hydrolysis and polycondensation of alkoxides to produce transparent glass without high-temperature processing. The resulting transparent glass has superior optical, mechanical, and thermal properties. In this paper, we describe the encapsulation of phycoerythrin into an optically transparent sol–gel matrix. Absorption and fluorescence measurements indicate that the protein retains its native activity in the sol–gel matrix. The effect of the sol–gel on the stability of the protein is determined through photodegradation experiments. In addition, we report the observation of a large two-photon induced fluorescence from PE in both solution and sol–gel glass. The potential applications of PE for two-photon-based three-dimensional scanning of biological fluorescence images and two-photon three-dimensional optical memory are discussed.

Methods

R-Phycoerythrin was purchased from Molecular Probes (Eugene, Oregon) without further purification. A tetramethoxysilane (TMOS, Aldrich) based sol–gel system was used for the protein encapsulation. The TMOS silica sol was prepared by mixing 0.1 mol of TMOS with 3.6 mL of 2.5 mM HCl. After sonicating for 25 min at 4 °C, the silica sol (1 mL) was mixed with 2 mL of phycoerythrin (50 μg/mL) in 0.01 M sodium phosphate buffer (pH 7). The PE-doped sol was then transferred to a cuvette. The sample gelled in about 2 min and was then stored at room temperature for aging and drying. During the aging process of 2 weeks, the PE-doped sol–gel sample was washed with deionized water three times to prevent the denaturation of protein due to the accumulation of alcohol. The aged gel was then exposed to ambient air for drying for about 2 weeks until about 70% (by weight) of the solvent had evaporated. The final PE concentration in sol–gel matrix is approximately 2.5×10^{14} molecules/cm^3.

The absorption spectra were taken using a spectrophotometer (Perkin-Elmer Lambda 9). The one-photon induced fluorescence spectra of PE in bulk sol–gel glass were taken with a fluorescence spectrophotometer (SLM 8000C, SLM Inc., NJ). The excitation wavelength was set at 490 nm.

Stability of the sol–gel sample was determined by measuring the photodegradation of the PE sol–gel samples using an argon ion laser as the light source for the photodegradation.

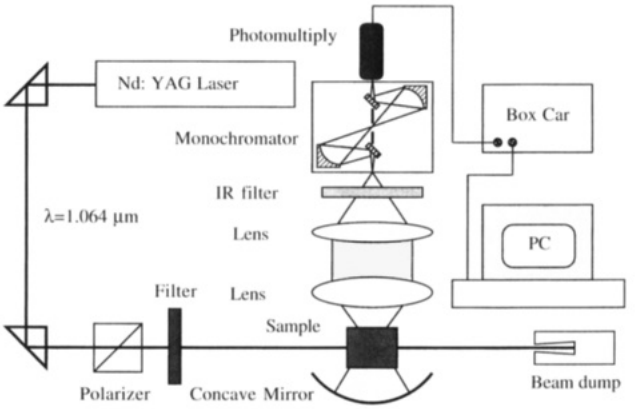

Figure 1. Schematic of the experimental setup for measuring the two-photon induced fluorescence spectrum of phycoerythrin.

The 514 nm laser beam was expanded to cover the entire sample, and the intensity of the laser beam was adjusted with a neutral density filter to about 30 mW/cm^2. The stability of PE in sol–gel glass was compared to that of PE in solution (100 mM NaCl, 100 mM sodium phosphate buffer). The photodegradation was carried out by illuminating both samples with the laser beam for about 30 min. The absorption spectra of these samples were then measured to determine the activity of the protein.

The experimental setup for the two-photon induced fluorescence measurement is shown in Figure 1. A Q-switched Nd:YAG laser with a 30-Hz repetition rate and 10-ns pulse width was used as the light source. The 1.06 μm IR beam with pulse energy of 10 mJ from the Nd:YAG laser was passed through the sample cell, and the two-photon induced fluorescence was collected with a focusing mirror and a condenser. The two-photon induced fluorescence passed through a monochromator and was measured by a photomultiplier tube. The scattering IR laser beam was blocked by a KG 3 filter, and the signal was averaged with a boxcar integrator and collected with a PC.

Results and Discussion

PE has a characteristic absorption band around 550 nm for light harvesting and a characteristic fluorescence band around 575 nm for energy transfer.[20] The light receptor portions of these proteins are open-chain tetrapyrroles that are coupled to the protein residues through thioether linkages. When this protein denatures, due to heat or other environmental changes, the absorption characteristics change and its fluorescence capability vanishes.[20] Absorption and fluorescence spectroscopy are therefore useful probes to monitor the effect of the encapsulation on the activity of the protein throughout the sol–gel process.

Figure 2 shows absorption spectra of PE at different stages of the sol–gel process, where Figure 2A is the absorption spectrum from PE-doped sol–gel measured 1 h after the sol–gel solution became gel; 2B is from aged gel; 2C is from dried gel. For comparison, the absorption spectrum of *R*-PE in solution is also shown (Figure 2D). The PE has two characteristic absorption peaks at 495 and 565 nm. Peak positions of the absorption spectra of PE in sol–gel glass and in solution are identical. This indicates that the absorption characteristics of the protein are preserved during the sol–gel process. However, there is a small change in the relative absorbance of the bands at 565 and 495 nm

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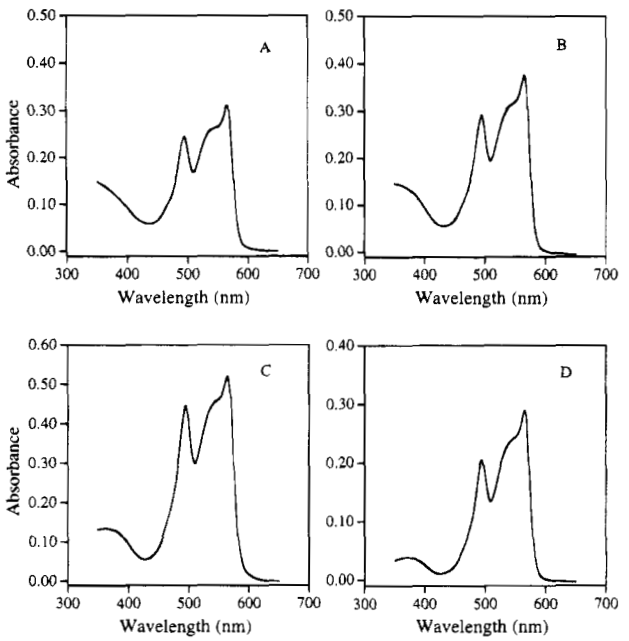

Figure 2. Absorption spectrum of PE in sol–gel matrix at different stages of sol–gel process (A–C) and in solution (D).

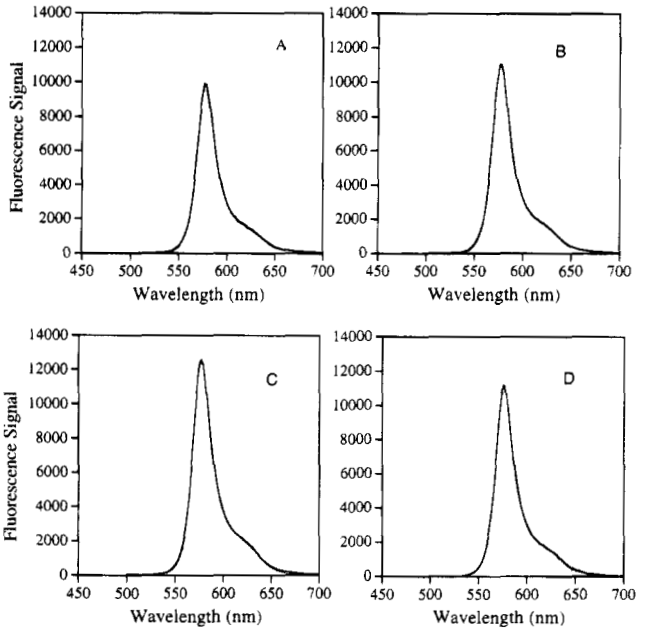

Figure 3. Fluorescence spectrum of PE in sol–gel matrix at different stages of sol–gel process (A–C) and in solution (D).

between PE in solution and in sol–gels. The absorbance at 565 nm diminished compared to the peak at 495 nm. Although part of this change can be attributed to the increased scattering in the sol–gel samples, the dried gel shows a decrease in the 565 nm band even after correction for scattering. It has been known that spectroscopic properties of PE are very sensitive to the aggregate state and conformation changes.[11] The study of biliprotein subunits, monomers, and higher aggregates has shown that interactions between biliproteins and their surrounding environment can induce conformation changes, which result in changes in the intensity of the long-wavelength absorption band.[11] The change in the relative intensity of the two bands can be attributed to possible conformation change due to the rigid sol–gel matrices.

Figure 3 shows the fluorescence spectra of PE at different stages of the sol–gel process. Figure 3A represents the fluorescence spectrum for PE doped sol–gel 1 h after gelation, Figure 3B for aged gel, and Figure 3C for aged and dried gel. For comparison, the fluorescence spectrum of phycoerythrin in solution is shown in Figure 3D. Because PE is known to lose its fluorescence capability upon degradation, the strong fluorescence signal observed is a clear indication that immobilized PE is active in the sol–gel matrix. The fluorescence emission spectrum of PE in solution peaks at 575 nm, while the spectrum of PE in sol gel is 2 nm red-shifted as compared to that in solution. It has been known that local environments surrounding the chromophore of the protein have a strong effect on the fluorescence spectrum.[21] The spectrum of PE is strongly influenced by the nearest-neighbor interactions, aggregation state, and conformation of the protein. The small red shifts of the fluorescence spectrum probably result from the protein conformational change due to the sol–gel matrix. Change in aggregation state and the polarization of the medium can also account for such a small shift. The fact that the shift is small suggests that the native conformation of the protein is not significantly altered by entrapment in sol–gel glasses.

The PE-doped sol–gel glass is optically transparent, and there is no aggregation of protein observed. The protein is dispersed in the sol–gel matrix homogeneously and looks very uniform optically as shown in the photograph in Figure 4. The glass on the right is the sample doped with PE and the one on the left is a dopant-free sol–gel glass. A blue beam (488 nm) from an argon ion laser was used to illuminate both samples. The laser beam first passed through the dopant-free sol–gel (blue line) and then illuminated the PE-doped sol–gel glass. The bright line on the PE-doped sol–gel is the fluorescence emission of the PE excited by the blue beam. The sol–gel glass is transparent, and the strong fluorescence beam can be clearly observed.

Although phycoerythrin is known as a light-harvesting protein and is stable under ambient light conditions, it has been found to denature under intense light illumination. Stability of PE toward photodegradation is very important for potential applications. Sol–gel matrixes provide a rigid cage that could enhance the stability of the entrapped molecule. Braun et al. reported the increased thermal stability of enzyme immobilized in sol–gel matrices.[15] Edmiston et al. used fluorescent probes to investigate the stability of albumin and myoglobin entrapped in sol–gel matrices.[22] Inhibition of photodegradation of luminescent inorganic molecules entrapped in sol–gel matrix has also been demonstrated.[23] However, no study on the photostability of light-transducing protein in sol–gel matrices has been reported to the best of our knowledge. Having demonstrated that PE remains active when incorporated in a sol–gel matrix, we next investigated the overall stability of PE under new environmental conditions. PE in both solution and sol–gel matrix was illuminated with an expanded laser beam. The absor-

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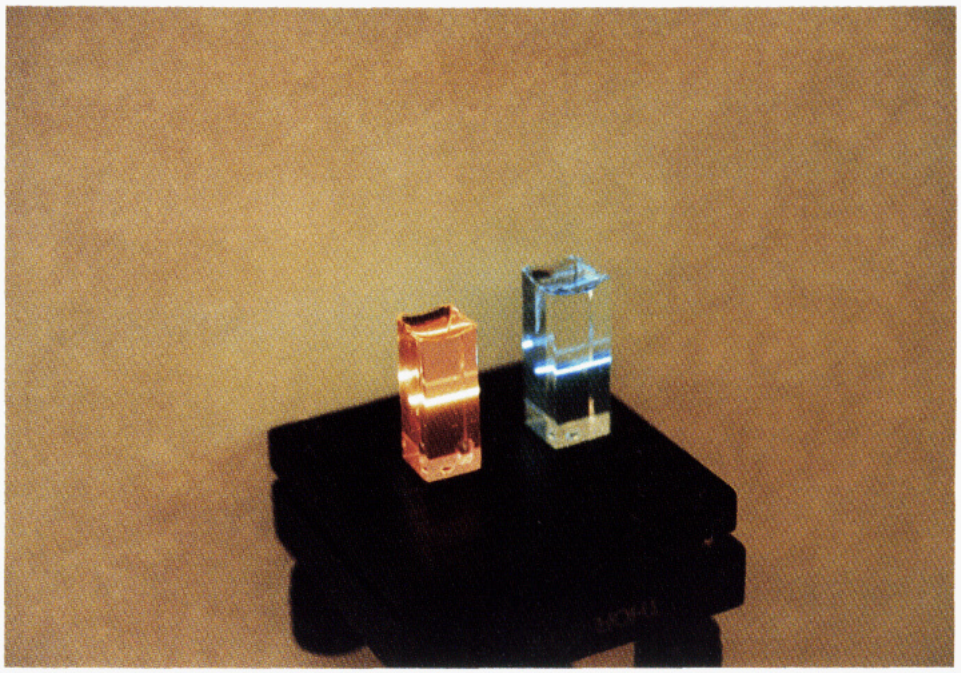

Figure 4. Photograph of sol–gel doped phycoerythrin and its fluorescence.

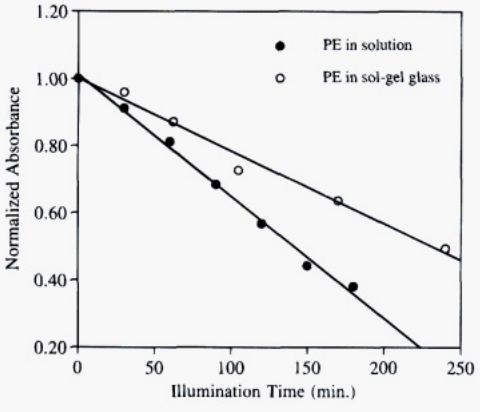

Figure 5. Absorbance at maxima of PE in solution (solid circle) and in sol–gel (open circle) as a function of the time that samples are illuminated with a 514 nm argon ion laser beam.

bances at the peak of the absorption band were measured as a function of the illumination time. The results of the studies are shown in Figure 5. The open circles represent the normalized absorbance as a function of illumination time for sol–gel encapsulated PE, and the solid circles represent PE in solution. The denaturation process was fitted by a linear curve, where the degradation rate was calculated from the slope of the curve. It was found that PE in solution degrades approximately 60% faster than that of the PE encapsulated in sol–gel. These results suggest that the sol–gel matrix not only retains the native activity of the PE but also enhances the stability of the protein toward photodegradation.

Phycobiliproteins are widely used as fluorescent tags in fluorescence immunoassay[12] because of their extremely high absorbance coefficients, intense fluorescence emission, and large Stokes shift. Although PE itself does not have molecular recognition capability, phycobiliprotein conjugates have been widely used in homogeneous energy-transfer immunoassays. For example, Kronick and Grossman have developed an immunoassay to determine the concentration of human IgG by using the energy transfer between the fluorescein-labeled human IgG and phycoerythrin-labeled antibody.[24] They have demonstrated that energy-transfer efficiency as high as 80% can be achieved. Sol–gel encapsulated PE conjugates may be used for energy-transfer immunoassay. A number of biosensors using sol–gel encapsulated biomolecules have already been reported.[17,25] The simplicity of sol–gel encapsulation together with the high stability of PE in sol–gel glass suggests that PE doped sol–gel glass is a promising material for biosensors.

Three-dimensional imaging of biological samples plays a crucial role in biomedical and biochemical research. Currently, confocal microscopy has been widely used for fluorescence imaging of biological samples with three-dimensional resolution. However, one problem encountered with the fluorescence image using the confocal technique is the photobleaching of the fluorescent probes. Recently, several groups have reported the use of two-photon induced fluorescence for the imaging of biological samples.[26,27] Two-photon induced fluorescence is a nonlinear process where the fluorescence signal depends quadratically on the excitation intensity and the emission is dominated by the most intense region of the excitation beam. This provides an optical sectioning effect which allows for fluorescence imaging with 3-D resolution without using the confocal configuration. Besides the inherent 3-D resolution obtained, two-photon fluorescence imaging is background free, and the photobleaching of the fluorescent molecules is minimized. However, the limitation to using such a technique for imaging is the necessity for the fluorescence tag to exhibit a large two-photon induced fluorescence. In addition, the fluorescent molecules must

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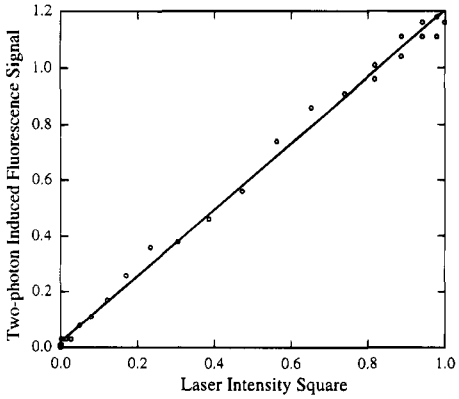

Figure 6. Fluorescence signal of PE as a function of the square of the 1.06 μm laser intensity.

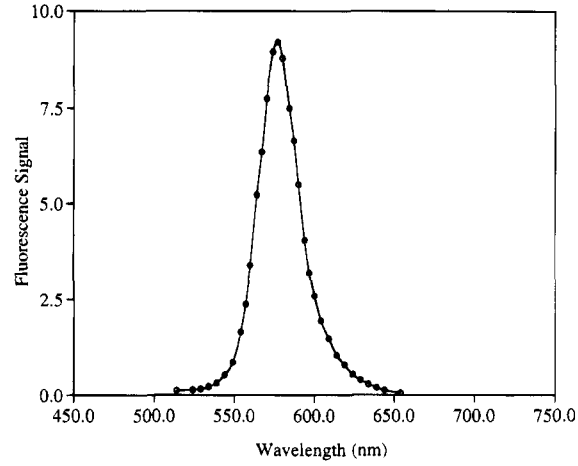

Figure 7. Two-photon induced fluorescence spectrum of PE-doped sol–gel glass.

remain relatively stable and exhibit minimum interference with the biological system to be imaged. PE is already widely used as a single-photon fluorescence tag for bimolecular research. However, to date no studies involving two-photon induced fluorescence from phycobiliprotein have been reported. We report the first measured two-photon fluorescence spectrum of PE in sol–gel using a 1.06 μm laser beam as excitation light (the results from PE in solution as well as from other phycobiliproteins will be published separately). A strong two-photon induced fluorescence is observed. Figure 6 shows the intensity dependence of the fluorescence signal, where the relative fluorescence intensity is plotted as a function of the square of the 1.06 μm laser intensity. The open circles are the measured fluorescence data, and the solid line is a linear fit. The linear fit of the data in Figure 6 shows that the fluorescence signal is proportional to the square of the laser intensity, which supports the proposed two-photon excitation mechanism. The two-photon induced fluorescence spectrum from PE in sol–gel is shown in Figure 7. It has an almost identical emission peak compared with that from one-photon induced fluorescence, with the exception that the shoulder at long wavelength is diminished in the two-phase case. The large observed two-photon induced fluorescence suggests that phycobiliproteins may find applications as fluorescence tags for two-photon fluorescence 3-D imaging.

Another potential application of the two-photon induced fluorescence from PE doped sol–gel glass is for three-dimensional optical memory. Three-dimensional optical memories that employ two-photon processes with two intersecting beams for writing and reading have already been proposed.[1,8,28,29] Two-photon fluorescence is one of the methods used to access data in a three-dimensional optical memory. Although a number of organic molecules have been proposed for such application,[28] materials based on biological molecules offer the advantage of tailoring and optimizing these properties through genetic engineering.[30] This has been illustrated by Hamp et al. with bacteriorhodopsin-based biomaterials where specifically designed mutants were found to improve the device performance.[31] The large observed two-photon induced fluorescence, enhanced stability, and the ability to specifically tailor the properties of PE, make sol–gel doped phycobiliprotein a potential material for 3-D optical memory.

Summary

We have developed a biomaterial by encapsulating PE in an optically transparent sol–gel glass. The absorption and fluorescence spectra indicate that protein activity is retained throughout the sol–gel process. In addition, the photodegradation experiment indicates that the encapsulated light-transducing protein is more stable against photobleaching than that in solution. For the first time the two-photon induced fluorescence of PE is reported. This observed two-photon induced fluorescence, coupled with the protein's inherent photochemical properties, relatively high stability, and its ability to be tailored through genetic engineering, make this material a promising system for potential applications in biomolecular sensing, imaging and information processing, and storage.

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