Cell-Free Synthesis, Functional Refolding, and Spectroscopic Characterization of Bacteriorhodopsin, an Integral Membrane Protein†

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Received September 29, 1993; Revised Manuscript Received October 25, 1993®

ABSTRACT: Bacteriorhodopsin (bR) is an integral membrane protein which functions as a light-driven proton pump in *Halobacterium halobium* (also known as *Halobacterium salinarium*). The cell-free synthesis of bR in quantities sufficient for FTIR and NMR spectroscopy and the ability to selectively isotope label bR using aminoacylated suppressor tRNAs would provide a powerful approach for studying the role of specific amino acid residues. However, no integral membrane protein has yet been expressed in a cell-free system in quantities sufficient for such biophysical studies. We report the cell-free synthesis of bacterioopsin, its purification, its refolding in polar lipids from *H. halobium*, and its regeneration with *all-trans*-retinal to yield bacteriorhodopsin in a form functionally similar to bR in purple membrane. Importantly, the yields obtained from *in vitro* and *in vivo* expression are comparable. Functionality of the cell-free expressed bR is established using static and time-resolved absorption spectroscopy and FTIR difference spectroscopy.

Recently, methods have been reported for the site-directed non-native amino acid replacement (SNAAR) of proteins using misaminoacylated suppressor tRNA (Bain et al., 1989, 1991; Noren et al., 1989; Mendel et al., 1991; Ellman et al., 1992). This technique, which requires protein synthesis in a cell-free expression system, could also be used for the site-directed isotope labeling (SDIL) of any atom in a protein, thereby extending the capability of several biophysical techniques to probe protein structure and function. For example, bands in the FTIR difference spectrum of an integral membrane protein which reflect changes in the structure, protonation state, and environment of individual amino acid residues (Rothschild et al., 1981, 1992; Gerwert, 1992; Rothschild, 1992; Rothschild & Sonar, 1994) could be directly assigned using SDIL. Distances between specific atoms in such proteins can also be determined using a combination of SDIL and rotational resonance NMR (Creuzet et al., 1991). Environments of specific residues can also be probed using SNAAR to incorporate amino acid analogs which serve as electron spin resonance (ESR) probes (Altenbach et al., 1989; Greenhalgh et al., 1991). These approaches, which rely on SDIL or SNAAR, would be especially valuable in the case of integral membrane proteins since only a few of these proteins have been crystallized for X-ray analysis.

Although the cell-free expression of integral membrane proteins has been used extensively to study protein translocation and protein folding (Wickner, 1980; Lingappa et al., 1984; Mize et al., 1986), large-scale cell-free expression and isolation has not been accomplished in quantities sufficient for FTIR and NMR measurements. Complications include the hydrophobicity of the nascent polypeptide and stringent requirements for correct refolding followed by posttranslational modifications. For example, *in vitro* synthesis of rhodopsin (Gurevich et al., 1989; Zozulya et al., 1990) requires proper glycosylation for folding and subsequent regeneration by retinal (Janssen et al., 1991). Continuous-flow methods have been reported for large-scale production of proteins (Spirin et al., 1988; Baranov et al., 1990; Kigawa & Yokoyama, 1991). However, these methods have not yet been demonstrated for integral membrane proteins.

Bacteriorhodopsin (bR) is part of a family of retinal-containing membrane proteins which includes halorhodopsin, a chloride pump, and sensory rhodopsin, a signal transducer involved in phototaxis (Stoeckenius & Bogomolni, 1982; Stoeckenius, 1985; Spudich & Bogomolni, 1988). Earlier work on bR has demonstrated that while halobacterial mRNA does not translate the apoprotein bacterioopsin (bOp) message when expressed in halobacterial S-40 extract (Gropp & Oesterhelt, 1989), it has been expressed in *Escherichia coli* S-30 extract as a fusion protein (McCoy & Khorana, 1983) and could also be translated in a wheat germ system in the presence of microsomal membranes (Bauer et al., 1992). However, cell-free expression of functional bR has not been reported.

Several features of bR make it an attractive candidate for cell-free synthesis, SDIL/SNAAR, and subsequent biophysical studies: (i) its folding in the membrane does not depend on the presence of a signal sequence; (ii) it is the only polytopic integral membrane protein that has been refolded after complete denaturation (Huang et al., 1981; Braiman et al., 1987; Popot et al., 1987); and (iii) advanced spectroscopic techniques have been developed, including time-resolved FTIR difference spectroscopy (Gerwert et al., 1990; Bousché et al., 1991, 1992; Braiman et al., 1991), resonance Raman spectroscopy (Lugtenburg et al., 1988; Mathies et al., 1991), and solid-state NMR (Smith et al., 1989; Creuzet et al., 1991; Metz et al., 1992), which provide information about bR function at the molecular level.

In this work, we demonstrate that bR can be synthesized in a cell-free system in a form functionally similar to bR in purple membrane and in quantities sufficient for biophysical measurements. Cell-free synthesis of bR is accomplished in an mRNA-dependent wheat germ extract. A simple purification protocol involving solvent extraction followed by refolding and regeneration in *Halobacterium halobium* lipids produces bR with normal absorption and light–dark adaptation properties. The functionality of the cell-free synthesized bR

† This work was supported by grants from the Army Research Office (DAAL03-92-G-0172), the NIH (GM47527), and the NSF (MCB9106017) to K.J.R.

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® Abstract published in *Advance ACS Abstracts,* December 1, 1993.

0006-2960/93/0432-13777$04.00/0

(cf-bR) is further established by using static and time-resolved absorption measurements as well as FTIR difference spectroscopy. These measurements demonstrate that the cf-bR undergoes all of the critical molecular changes which are found in native bR during its photocycle.

MATERIALS AND METHODS

All chemicals used were obtained from Sigma unless otherwise mentioned. Restriction enzymes were purchased from New England Biolabs. In order to place the *bop* gene under control of the SP6 promoter, plasmid pSP72-*bop* was constructed by inserting the *bop* gene (*Hin*dIII–*Eco*RI fragment) from the cloning vector pSBO2 (Karnik et al., 1987) into the multiple cloning site of vector pSP72 (Promega Corp., Madison, WI).

In Vitro Transcription To Obtain bop mRNA. Large-scale mRNA synthesis was carried out by *in vitro* transcription using the SP6-Ribomax system (Promega Corp.) from pSP72-*bop* linearized by *Eco*RI digestion at the 3′-end of the *bop* gene.

Cell-Free Synthesis of Bacterioopsin in Wheat Germ Extracts. Cell-free synthesis of bacterioopsin was carried out in a mRNA-dependent wheat germ translation system (Promega Corp.). Synthesis was continued for 3 h at 25 °C according to protocols provided. The cell-free synthesis of bOp was analyzed by SDS–PAGE (12%) followed by autoradiography using [^{35}S]methionine incorporation (Amersham Corp., Arlington Heights, IL; 800 Ci/mmol at 16 mCi/mL). Synthesis of bOp was optimized with respect to mRNA, K^+ concentration, and time of reaction using either SDS–PAGE/autoradiography or [^{35}S]methionine incorporation assay after TCA precipitation.

Purification of Bacterioopsin after Cell-Free Synthesis. After a typical synthesis of bOp in a 2-mL reaction mixture, its purification was carried out using a solvent extraction method (Braiman et al., 1987) except with some modifications developed to handle the small amount of cf-bR produced, requiring micromanipulations. To the reaction mixture were added methanol (4 vol), chloroform (2 vol), and water (3 vol) to induce interphase precipitation. The precipitated protein was extracted with a chloroform:methanol:water:triethylamine system (Braiman et al., 1987). After the total protein concentration of the solvent extract was measured (Braiman et al., 1987), SDS was added (protein:SDS ratio, 1:5), the solvent mixture was lyophilized, and the product was finally redissolved in water (protein concn, 2 mg/mL). Insoluble protein fractions were then removed by centrifugation.

Refolding, Regeneration, and Reconstitution of Cell-Free Synthesized Bacterioopsin. Bacterioopsin in SDS solution was refolded in polar lipids from *H. halobium* and regenerated by exogenous addition of *all-trans*-retinal using procedures described previously (Popot et al., 1987) in a final volume of 100 μL. Regenerated samples were dialyzed for 2 days against 500 mL of "K-buffer" (30 mM sodium phosphate and 150 mM KCl) at room temperature. Further purification consisted of several washes with K-buffer. Chromophore regeneration was determined on the basis of absorption at 558 nm.

Static and Time-Resolved Visible Absorption of Cell-Free Synthesized Bacteriorhodopsin. The static visible absorption spectra of the cf-bR were measured in a 100-μL submicro cell (type 16.100; Starna Cells Inc., Atascaredo, CA) using a UV–visible Schimadzu 2101 spectrophotometer (Schimadzu Corp., Kyoto, Japan) equipped with a 60-mm integrating sphere which reduced absorption loss due to light scattering (Sonar et al., 1993). Light adaptation was carried out as described previously (Sonar et al., 1993).

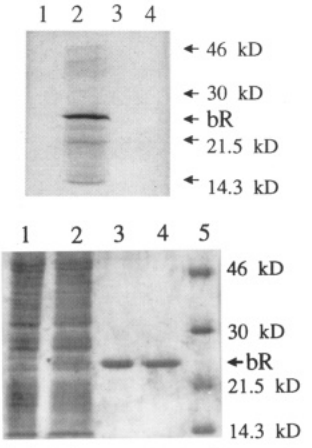

FIGURE 1: (A, left) Cell-free synthesis of bacterioopsin. *bop* mRNA was expressed in wheat germ extracts using [^{35}S]methionine labeling. Lane 1, no mRNA; lane 2, synthesis of bacterioopsin in response to added *bop* mRNA; lane 3, pure bacterioopsin (arrow indicates position of bR); lane 4, molecular weight markers (as marked). Synthesis in wheat germ (Promega Corp., Madison, WI) was carried out for 3 h at 25 °C according to protocols provided. SDS–PAGE was carried out in a 12% gel and was followed by autoradiography. (B, right) Coomassie blue-stained SDS–PAGE analysis during purification of bOp after cell-free synthesis. Lane 1, total wheat germ reaction mixture after bOp synthesis; lane 2, solvent-extracted proteins from wheat germ reaction mixture; lane 3, bacterioopsin after refolding in halobacterial lipids three washes with phosphate buffer (30 mM sodium phosphate, 150 mM KCl) by centrifugation (14000*g*, 10 min, room temperature); lane 4, *in vivo* expressed and purified bR; lane 5, molecular weight markers (as marked).

Time-resolved difference spectra were measured using a gated optical multichannel analyzer (1420 UV-enhanced optical multichannel analyzer, Model 1460 controller; Princeton Applied Research, Princeton, NJ) and 532-nm pulsed excitation from a frequency-doubled Nd:YAG laser (DCR-11, Spectra Physics, Mountain View, CA) (40 mJ/pulse laser output, 7-ns pulse width) (Sonar et al., 1993). The data-acquisition time was varied from 100 ns to 100 μs in order to optimize the signal-to-noise ratio. The delay times given refer to the time between the laser flash and the initiation of the data-acquisition period.

FTIR Difference Spectroscopy. bR→K and bR→M difference spectra were recorded as described previously (Roepe et al., 1987; Braiman et al., 1988). Samples in distilled water were prepared by air drying approximately 15 μg/cm^2 of sample on a AgCl window and then rehydrating prior to insertion into a sealed transmission cell which was mounted in a Helitran cryostat (Air Products, Allentown, PA). Samples were light-adapted at room temperature prior to cooling by illuminating the sample for at least 15 min with a 150-W tungsten light source equipped with a 505-nm long-pass filter. Spectra were recorded at 2 cm^{-1} resolution using a Nicolet Analytical Instrument 740 spectrophotometer equipped with an MCT detector (Madison, WI).

RESULTS AND DISCUSSION

Figure 1A shows that translation of *bop* mRNA in a wheat germ extract in the presence of [^{35}S]methionine results in production of a protein which migrates at the same position (26 kDa) as bR (Figure 1A, lanes 2 and 3).[1] This synthesis

[1] Other bands appearing in lane 2 (Figure 1A) correspond to translation products of non-bOp mRNAs formed as a result of nonspecific initiations during the run-off transcription.

was found to be optimal at an mRNA concentration of 40 μg/mL and a K^+ concentration of 90 mM (data not shown).

Figure 1B shows the results of purification as analyzed by SDS–PAGE followed by Coomassie staining. After 3 h of synthesis, the total wheat germ extract shows a heterogeneous mixture of proteins (lane 1). At this stage, it is difficult to identify a band due to nascent bOp. However, after solvent extraction (lane 2) a distinct band is found corresponding to the mobility of bOp, although many other proteins are still present in the extract. In order to further purify cf-bR, solvent-extracted proteins were transferred to SDS as described in Materials and Methods. As shown previously, SDS is important for promoting α-helical structure, and in the case of bOp it is found to be important for *in vitro* refolding after its denaturation (Huang et al., 1981). Bacterioopsin in SDS solution was then refolded in polar lipids from *H. halobium* and regenerated using *all-trans*-retinal (see below). Further purification consisted of several washes with a phosphate buffer (30 mM sodium phosphate and 150 mM KCl). As shown in Figure 1B, these steps result in almost complete purification of bR, as indicated by a single band (lane 3) corresponding to bR (lane 4).

It is worth noting that, in contrast to the experiments of Braiman et al. (1987), column chromatography was avoided in the purification, mainly because of the very small quantities and hydrophobicity of the nascent bOp polypeptide. In addition, instead of the refolding and regeneration of purified nascent bOp being done in DMPC/CHAPS micelles, it was done directly in polar lipids from *H. halobium*. The refolding in halobacterial lipids has been shown to result in reconstitution of bR into fragments similar to purple membrane (Popot et al., 1987), an important requirement for FTIR difference spectroscopy (see below). In addition, this method results in effective purification of bR since repeated washings with high-salt buffer separate the heavier bR membrane fragments from other soluble contaminating proteins.

Regeneration of cell-free synthesized bOp by exogenous addition of *all-trans*-retinal results in formation of a purple complex absorbing at 560 nm, characteristic of dark-adapted bR (bR_{560}) (Figure 2A, curve 1). On the basis of the extinction coefficient at 558 nm (54 000 M^{-1} cm^{-1}), the total yield for cell-free synthesis of bR is found to be 24 μg/mL. This yield corresponds to 24 mg of bR per liter of reaction mixture, comparable to that obtained from *H. halobium* cultures (Oesterhelt & Stoeckenius, 1974). Curves 2 and 3 (Figure 2A) show that cf-bR undergoes a normal light adaptation upon illumination causing a shift in its visible absorption maximum (λ_{max}) from near 560 to 570 nm to form the light-adapted species (bR_{570}). Thus the present method effectively purifies, refolds, and regenerates small quantities of nascent bOp polypeptide to give functional bR suitable for FTIR and other measurements.

In order to check the functionality of the cf-bR, we studied its photocycle. Upon light absorption by bR_{570}, the K intermediate is formed, which thermally relaxes to the L, M, N, and O intermediates and then returns to bR_{570} (Lozier et al., 1992). Figure 2B shows the time-resolved visible absorption difference spectra of cf-bR, which are very similar to those of native bR (Sonar et al., 1993). Decay of the K intermediate (reduction in intensity near 615 nm), formation of the M intermediate (rise of intensity at 412 nm), and decay of the M intermediate (decay of the 412-nm band) are clearly seen. The kinetics of each of these processes was very similar for native (Sonar et al., 1993) and cell-free expressed bR (K decay = 0.40 vs 0.43 μs; M rise = 24.0 vs 23.8 μs; M decay = 5.4 vs 5.7 ms). It should be noted that the visible absorption of these intermediates and their formation and decay kinetics in the photocycle are highly sensitive to the environment of bR, including crystallinity (Varo & Lanyi, 1991b), lipid species (or detergents) (Milder et al., 1991), temperature (Tsuda et al., 1983; Lin et al., 1991), hydration level (Varo & Lanyi, 1991a), pH, and salt concentration (Kouyama et al., 1988; Varo & Lanyi, 1989). Changes in the bR structure induced by site-directed mutations can also cause drastic changes in its properties (Khorana, 1988).

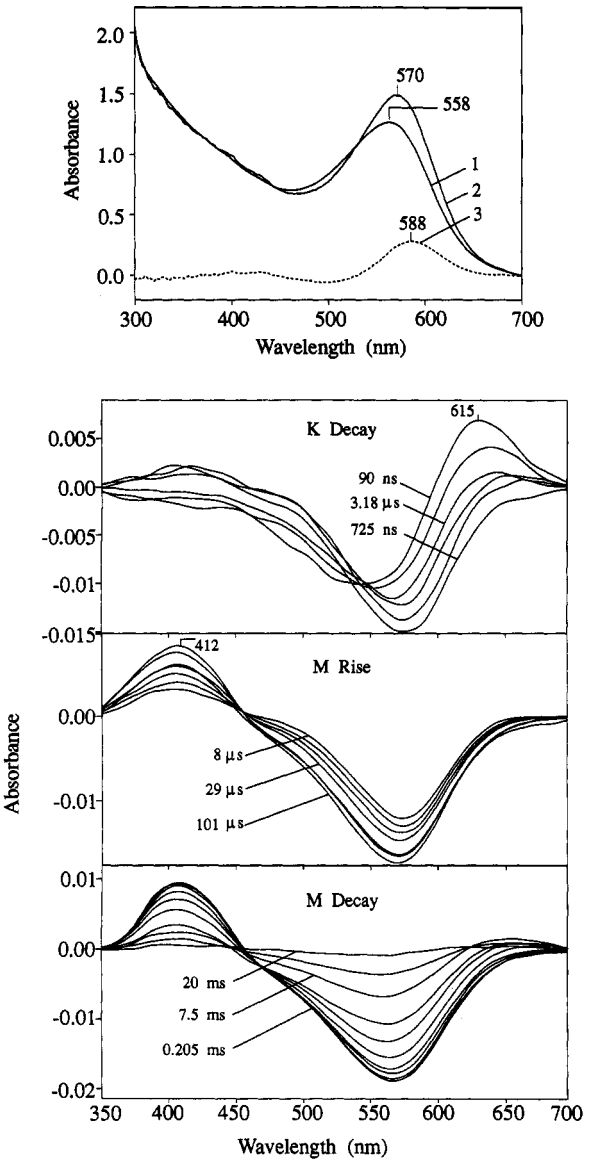

FIGURE 2: (A, upper panel) Refolding and regeneration of cell-free synthesized bacterioopsin: absorption spectra after refolding and regeneration of cell-free synthesized bacterioopsin in halobacterial lipids. Curve 1, dark-adapted; curve 2, light-adapted; curve 3, difference spectrum obtained by subtracting dark-adapted spectrum (curve 1) from light-adapted spectrum (curve 2), which is indistinguishable from that obtained for bR (Sonar et al., 1993). (B, lower three panels) Characterization of the photocycle of cell-free synthesized bacterioopsin. Time-resolved difference spectra were measured as described previously (Sonar et al., 1993). The delay times given refer to the time between the laser flash and the data-acquisition period. Difference spectra were obtained at (top) K decay (0.09, 0.38, 0.725, 1.24, 2.0, and 3.18 μs). (middle) M rise (8.0, 12.5, 19.2, 29.3, 43.0, 67.0, and 101 μs), and (bottom) M decay (0.205, 0.349, 0.589, 0.986, 1.6, 2.7, 4.5, 7.5, 12.5, and 20 ms).

The absolute FTIR absorption spectra of native bR and the cf-bR are shown in Figure 3A. The frequency, intensity, and dichroism of the two intense bands at 1659 cm^{-1} (amide I)

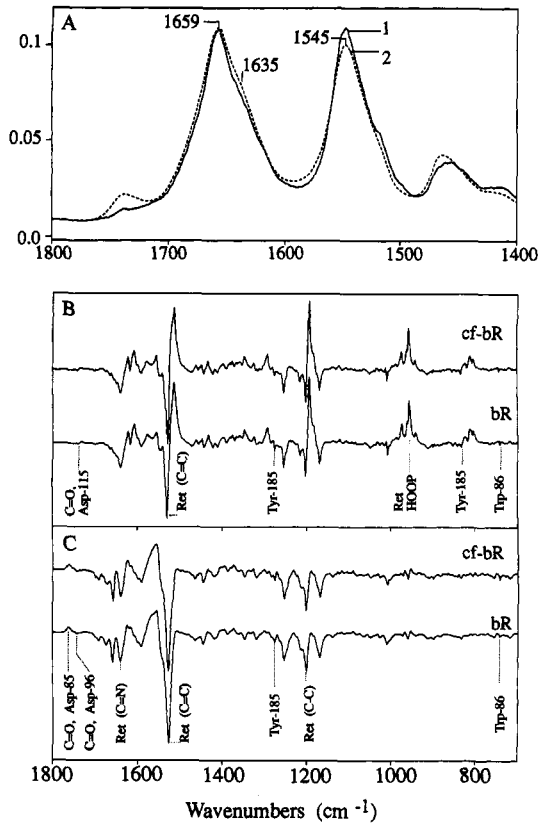

FIGURE 3: FTIR difference spectroscopy of cell-free expressed bR. FTIR absorbance and FTIR difference spectra were recorded at 2 cm^{-1} resolution. (A) FTIR absolute absorbance spectra of cell-free synthesized bR (cf-bR) after refolding and purification (- - -) and native bR (—). (B, C) Comparison between FTIR difference spectra of cf-bR and native bR for bR→K transition measured at 80 K (B) and bR→M transition measured at 250 K (C). For further details on band assignments, see Rothschild (1992).

and 1545 cm^{-1} (amide II) are extremely sensitive to protein secondary structure. In the case of bacteriorhodopsin, they reflect a predominantly α-helical structure which has a net orientation transverse to the membrane plane (Rothschild & Clark, 1979). The similar appearance of these bands in both samples indicates that the native bR and cf-bR have very similar structures. For example, we can rule out the existence of high levels of unfolded bOp which would give rise to a prominent shoulder near 1635 cm^{-1}.

The FTIR difference spectrum reflects the structural changes which occur in the retinal chromophore, protein backbone, and individual amino acid residues during the different steps in the bR photocycle (Rothschild et al., 1981, 1992; Gerwert, 1992; Rothschild, 1992; Rothschild & Sonar, 1994). As shown in panels B and C of Figure 3, the bR→K and bR→M FTIR difference spectra of native bR and cf-bR are almost identical. Bands previously assigned on the basis of site-directed mutagenesis to vibrations of specific protein residues can be identified in the cf-bR sample, including bands at 742 (Trp-86), 1277 (Tyr-185), 1760 (Asp-85), 1730/1740 (+/−) (Asp-115), and 1742 cm^{-1} (Asp-96) (Braiman et al., 1988; Gerwert, 1992; Rothschild, 1992; Rothschild et al., 1992; Rothschild & Sonar, 1994). Vibrations assigned to the retinal chromophore are also unchanged in the cell-free bR sample. *Thus, we conclude that the cell-free synthesized bR is fully functional and undergoes normal structural changes during its photocycle.* Furthermore, less than 2 mL of wheat germ translation reaction mixture is sufficient to obtain a cf-bR sample which yields high-quality FTIR difference spectra.

CONCLUSIONS

We have demonstrated that it is possible to synthesize bR in a cell-free system, purify it using a simple solvent extraction technique, and refold and regenerate it with native halobacterial lipids and *all-trans*-retinal to yield intact purple membrane. The functionality is demonstrated by static and time-resolved visible absorption and FTIR difference spectroscopy. The ability to produce significant levels of functional bR by cell-free synthesis should open several new avenues for biophysical studies. For example, uniform isotopic labeling of a specific amino acid in bR, which is impeded by intracellular amino acid biosynthetic pathways during *in vivo* expression, can be accomplished by cell-free expression. Such uniform labeling is important for both FTIR and solid-state NMR studies. Site-directed isotope labeling, which requires at present the use of cell-free synthesis, allows direct assignment of bands to the vibrations of individual amino acid residues (S. Sonar, C.-P. Lee, M. Coleman, N. Patel, X. Liu, T. Marti, H. G. Khorana, U. L. RajBhandary, and K. J. Rothschild, manuscript in preparation). This method completely avoids structural perturbations introduced by site-directed mutagenesis, especially when the mutations involve active site residues. In general, questions regarding the state of individual amino acid residues inside a protein and their dynamics during its function could be addressed directly by using these approaches.

ACKNOWLEDGMENT

We thank U. L. RajBhandary, H. G. Khorana, C-P. Lee, and Guilford Jones, Jr., for advice and review of the manuscript. We also thank H. G. Khorana and T. Marti for the gift of synthetic *bop* gene and for assistance in construction of the pSP72-*bop* plasmid. We thank O. Bousché for excellent technical assistance.

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