Heterogeneous Rieske Proteins in the Cytochrome $b_6f$ Complex of Synechocystis PCC6803?*

The completely sequenced genome of the cyanobacterium Synechocystis PCC6803 contains three open reading frames, petC1, petC2, and petC3, encoding putative Rieske iron-sulfur proteins. After heterologous overexpression, all three gene products have been characterized and shown to be Rieske proteins as typified by sequence analysis and EPR spectroscopy. Two of the overproduced proteins contained already incorporated iron-sulfur clusters, whereas the third one formed unstable aggregates, in which the FeS cluster had to be reconstituted after refolding of the denatured protein. Although EPR spectroscopy showed typical FeS signals for all Rieske proteins, an unusual low midpoint potential was revealed for PetC3 by EPR redox titration. Detailed characterization of Synechocystis membranes indicated that all three Rieske proteins are expressed under physiological conditions. Both for PetC1 and PetC3 the association with the thylakoid membrane was shown, and both could be identified, although in different amounts, in the isolated cytochrome $b_6f$ complex. The considerably lower redox potential determined for PetC3 indicates heterogeneous cytochrome $b_6f$ complexes in Synechocystis and suggests still to be established alternative electron transport routes.

The functional core of cytochrome $bc$ complexes consists of three subunits, which are a $b$-type cytochrome, a $c$-type cytochrome, and the Rieske iron-sulfur protein (1). In contrast to the cytochrome $bc_1$ complex of mitochondria and bacteria with three subunits, cytochrome $b$, cytochrome $c_1$, and the Rieske protein, the homologous cytochrome $b_6f$ complex of chloroplasts and cyanobacteria contains four core subunits named cytochrome $b_5$, subunit IV, cytochrome $f$, and the Rieske protein (2).

The Rieske protein consists of an N-terminal transmembrane $\alpha$-helix, which anchors the protein in the membrane (3, 4), and a large soluble domain on the luminal side of the membrane. The three-dimensional structure of this luminal part has been solved both for the Rieske protein of a cytochrome $b_6f$ complex (5) and of a cytochrome $bc_1$ complex (6) to a resolution of 1.8 and 1.5 Å, respectively. The redox active [2Fe-2S] cluster, which is bound by the luminal domain, is ligated by two cysteine and two histidine residues with one iron being ligated by the two cysteines and the other by the two histidines. It is likely that this unusual coordination causes the high midpoint potential of about +100 to +400 mV. Besides these coordinating amino acids there are two additional cysteine residues, which form a disulfide bridge close to the iron-sulfur cluster. In the amino acid sequence the coordinating amino acids as well as the additional cysteines are organized in two boxes with highly conserved sequences in all cytochrome $bc$ complexes. It was shown that substitutions of single amino acids within or around these boxes affect the midpoint potential of the Rieske proteins (7) and that this midpoint potential can be predicted to some extent from the amino acid sequence. Proteins with the sequence CPCHGSX of the C-terminal iron-sulfur cluster-binding site (Box II) oxidize high potential quinones like ubi- and plastoquinone, and these proteins have a midpoint potential between +250 and +375 mV. In contrast, in organisms utilizing menaquinone, one or both of the lateral residues Ser and Tyr are replaced by nonhydroxylated amino acids resulting in a lowered midpoint potential of the Rieske protein. The midpoint potentials of these menaquinone-oxidizing Rieske proteins are in the range of +100 to +180 mV. Whereas Rieske cluster of cytochrome $b_6f$ complexes have been found to have midpoint potentials in the range of about +270 to +360 mV, in cytochrome $bc_1$ complexes also proteins with a lower midpoint potentials can be found.

Although heterologous overexpression of proteins is a routine method to produce large amounts of a protein, the overexpression of membrane proteins in Escherichia coli often leads to cell death due to the toxicity of the foreign protein (8). There are, however, reports on the successful overexpression of Rieske proteins. The heterologous overexpression of the Rieske protein from the cyanobacterium Nostoc PCC7906 resulted in copious quantities of the protein located in inclusion bodies (9). Also, the previously described overexpression of the Synechocystis Rieske protein PetC1 yielded predominantly a non-native form clustered in inclusion bodies (10). However, both studies agree that a small fraction of the overexpressed protein can be found in E. coli membranes, although without incorporated iron-sulfur cluster. A successful overexpression of a functional Rieske protein with incorporated iron-sulfur cluster was reported for two proteins from hyperthermophilic archaea (11, 12), which was not achieved up to now with “mesophilic” Rieske proteins.

The completely sequenced genome of the cyanobacterium Synechocystis PCC6803 contains three open reading frames named sll1316 (petC1), sll1185 (petC2), and sll1182 (petC3) encoding for three putative Rieske iron-sulfur proteins. The characterization of the overexpressed proteins presented in this paper reveals that all three petC genes encode functional Rieske proteins. Although two proteins, PetC1 and PetC2, are

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rather similar, the encoded protein PetC3 displays different structural and functional properties. In this report the functional expression of the three petC genes in the cyanobacterium Synechocystis PCC6803 is shown, and at least two PetC proteins were found to be associated with the cytochrome b6f complex of this organism.

MATERIALS AND METHODS

Growth Conditions—Synechocystis PCC6803 wild type and mutant strains were grown at 30 °C in BG11 medium according to Ref. 13. Constant illumination was provided by white fluorescent lamps with a photon flux density of 40 μmol photons m−2 s−1.

E. coli strains were grown in broth or agar LB, supplemented by 100 μg of ampicillin/ml or 30 μg of chloramphenicol/ml where indicated. Strain DH5α was used for plasmid propagation and BL21(DE3) for heterologous overexpression of the Rieske proteins.

DNA Techniques—Molecular cloning was carried out using standard techniques as described previously (14). Enzymes used for PCR and cloning were obtained from MBI Fermentas. PCR was carried out using a Bio-Rad thermocycler. DNA sequencing was performed by MWG Biotech (Ebersberg, Germany). For PCR, genomic Synechocystis DNA was prepared according to Ref. 15.

Overproduction of the Rieske Proteins—Heterologous overexpression of the three petC genes of Synechocystis was done by cloning them separately into the expression plasmid pRSET6a (16). The three genes were amplified by PCR using the primers NPTpetC1 (gggaattccatatggatccttaggctttgaccagaatgttgttgc) and CTPetC3 (gcgatccttaggctttgaccagaatgttgttgc) to amplify petC3. For cloning of the PCR-amplified full-length Rieske genes, a NdeI side was introduced at the 5’ end of the gene including the translation start codon ATG, and a BamH1 or BglII side was added at the 3’ terminus of petClpetC2 and petC3, respectively. The PCR-amplified genes were cleaved with NdeI and BamHI/BglII and cloned into the appropriately restricted expression plasmid pRSET6a. After transformation of the expression plasmids into E. coli BL21(DE3), the expression cultures were grown to an A660 = 0.6 at 37 °C in LB media containing 100 μg of ampicillin/ml. Expression of the foreign genes was induced by addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested after 4 h of incubation, resuspended in buffer PBS (150 mM NaCl, 2.5 mM KCl, 13 mM NaHPO4, 1.8 mM KH2PO4, pH 7.2), and broken by sonication. Soluble E. coli proteins and membranes were separated from insoluble components (“low speed pellet”) by centrifugation at 10,000 × g. E. coli membranes were isolated by centrifugation of the supernatant at 100,000 × g (“high speed pellet”).

Spectroscopic Methods—EPR spectra of the isolated cytochrome b6f complex and membranes were recorded on a Bruker EPR200 spectrometer, the sample temperature was kept at 15 K with a helium cryostat. Other parameters for the measurements include 6.3 milliwatts of microwave power, 10 G modulation amplitude, and 9.44-gigahertz microwave frequency. E. coli membranes with the incorporated iron-sulfur proteins were adjusted to pH 5.5 and a protein concentration of 40 mg/ml. EPR-monitored redox titrations were done as outlined previously (18).

Protein Analysis—Membranes of the cyanobacterium Synechocystis PCC6803 were isolated as described previously (17), and the cytochrome b6f complex was extracted from the thylakoids by incubation with 1% (w/v) n-dodecyl-β-D-maltoside for 30 min at room temperature. After centrifugation at 40,000 × g, the cytochrome b6f complex was purified by two high performance liquid chromatography steps according to Wenk et al. Characterization of the complex by SDS-PAGE and immunoblotting revealed the presence of cytochrome f (29 kDa), cytochrome b6 (24 kDa), the Rieske protein (19 kDa), subunit IV (17 kDa), and several small subunits in the range of 3–8 kDa. Further characterization of the complex by gel filtration chromatography showed that the isolated complex was monomeric.

Components of Synechocystis PCC 6803 membranes or the purified cytochrome b6f complex were separated by 15% SDS-PAGE gels (19).

FIG. 1. Sequence alignment of the three Rieske proteins encoded in the genome of Synechocystis PCC6803. The iron-sulfur binding sites (Box I and II) are marked. Parts of the protein sequences that have been used for the production of peptide antibodies are underlined. Amino acids predicted to form a transmembrane α-helix are printed in boldface letters.
Although up to now no information is available on the three proteins, their sequences indicate binding sites for the typical Rieske FeS cluster.

Also, the N-terminal part of all three protein sequences consists of hydrophobic regions of about 20 amino acids, which are predicted to form a α-helical transmembrane anchor.

Production of Specific Peptide Antibodies—Short amino acid sequences of the individual Rieske proteins were selected for the production of specific peptide antibodies in rabbits (Fig. 1), and the raised antibodies were tested for their specificity. Each antibody cross-reacts with the individual Rieske protein containing the peptide sequence used for immunization. Whereas the anti-PetC1 and anti-PetC2 antibodies showed no cross-reactivity with any of the other two Rieske proteins, the anti-PetC3 antibody also cross-reacts with the PetC1 protein. Because of the molecular mass difference between these two proteins, the identification of the cross-reacted protein was straightforward.

Heterologous Overexpression and Characterization of the Isolated Putative Rieske Proteins—To facilitate more detailed studies on the three isolated Synechocystis proteins, plasmids for the heterologous overexpression of the three full-length proteins in E. coli were constructed as described under “Materials and Methods.” All three E. coli strains carrying the expression plasmids for PetC1, PetC2, and PetC3, respectively, show a predominantly expressed protein with a apparent molecular mass of 19 (Fig. 2, A and B) or 14 kDa (Fig. 2C); these masses agree well with the molecular weight derived from the amino acid sequences of the individual Rieske proteins. The different Rieske antibodies cross-reacted as expected with the overexpressed proteins from E. coli (Fig. 3), and they showed no cross-reactivity with fractions of E. coli that did not contain these overexpressing constructs. Whereas most of the heterologous protein was deposited in inclusion bodies (low speed pellet), a minor fraction of the overexpressed protein was found to be associated with E. coli membranes (high speed pellet).

Although the inclusion body fractions showed only a very weak Rieske-type EPR signal (results not shown), membranes isolated from cells in which PetC2 and PetC3 were overexpressed yielded a typical Rieske-type EPR signal as shown in Fig. 4, A and B, respectively. This signal was not observed with membranes from control cells (Fig. 4C). Interestingly, in the control membranes an EPR signal of unknown origin with a g value of 2.02 was detected, most likely caused by the presence of small amounts of copper. Besides this signal, membranes of the E. coli strains overexpressing PetC2 showed additional signals with gₓᵧᵧ = not detected, 1.90, and 2.03 (Fig. 4A). In membranes of cells overexpressing PetC3, a Rieske-type EPR signal with a greater amplitude was detected, and the gₓᵧᵧ value overlaps considerably with the signal of the unknown origin. However, the detected gₓᵧᵧ values of 1.78, 1.90, and 2.02 (Fig. 4B) also indicate the presence of a typical Rieske iron-sulfur cluster. Because PetC1 could not be obtained in a native form with an incorporated iron-sulfur cluster, this protein was purified from inclusion bodies of E. coli cells overexpressing this protein. After solubilization by urea, the inclusion body fraction
was further purified by anion exchange high pressure liquid chromatography as described previously (10). Incorporation of the iron-sulfur cluster into the refolded protein was achieved enzymatically using IsnS, which is known to mediate the incorporation of iron-sulfur clusters, and the reconstituted protein was further characterized (10). The isolated PetC1 protein showed $g$ values of $K_{ox} = $ not detected, 1.90, and 2.04, which are identical to the EPR characteristics of the isolated cytochrome $b_{c2}$ complex (10), whereas the $g$ values of the isolated PetC2 and PetC3 proteins show slight deviations.

**Determination of the Midpoint Potential of Three Synechocystis Rieske Proteins by EPR Spectroscopy**—In contrast to PetC1 and PetC2, which were isolated in the fully reduced form after overexpression, PetC3 was almost completely oxidized under aerobic conditions when no reducing agents were added. This indicates a lower redox midpoint potential ($E_{m}$) of this protein in comparison with the other two proteins. Determination of the midpoint potentials of the two Rieske proteins PetC1 and PetC3 by redox titration (Fig. 5, A and B) yielded a high $E_{m}$ value of +320 mV for PetC1, which is in good agreement with values of other Rieske proteins from cytochrome $b_{c2}$ and $b_{1}$ complexes. Interestingly, the midpoint potential of the third Rieske protein, PetC3, was determined to +135 mV (Fig. 5B), i.e. about 200 mV lower than the midpoint potential of the PetC1 protein of *Synechocystis*. This fact might explain the observed oxidized form of PetC3 during the EPR measurements under aerobic conditions in the absence of reductants. All attempts to determine the exact value of the midpoint potential of PetC2 by EPR failed. The purified protein was not stable after addition of the mediators, as already observed for other redox active proteins.\(^3\) However, based on other methods the midpoint potential of PetC2 could be roughly estimated (see “Discussion”).

**Phylogenetic Analysis**—Phylogenetic analysis of the three Rieske proteins showed that PetC1 is very closely related to most other cyanobacterial Rieske proteins (Fig. 6). Although PetC2 is more distant to most of the cyanobacterial proteins, it also clearly belongs to the group of cytochrome $b_{c2}$ complex Rieske proteins. In contrast, PetC3 shows no particular similarities to other cytochrome $b_{c2}$ complex Rieske proteins. This protein segregates into a heterogeneous group of proteins, which includes the Rieske proteins from *Helioibacillus*, *Myco- bacterium*, and *Streptomyces* as well as the archaeal Rieske proteins (Fig. 6). This phylogenetic classification and the detected low midpoint potential argue against a functional role of this protein in the cyanobacterial cytochrome $b_{c2}$ complex.

**Translational Analysis**—Although the characterization of the heterologously overexpressed proteins showed that all three proteins are Rieske iron-sulfur proteins, the expression of all three gene products in *Synechocystis* had to be demonstrated. Polyclonal anti-peptide antibodies raised against unique regions of the proteins (Fig. 1) were used to test *Synechocystis* membranes for the presence of the three gene products. Fig. 7 shows an immunoblot of membranes from *Synechocystis* WT,\(^4\) ΔpetC1, ΔpetC2, and ΔpetC3. The anti-PetC1-antibody reacted with a protein of 19 kDa in WT, ΔpetC2, and ΔpetC3 membranes. By using membranes from the ΔpetC1 strain no cross-reaction was observed, indicating the monospecificity of this antibody. The anti-PetC3-antibody reacted with proteins with an apparent molecular mass of 19 and 14 kDa caused by the cross-reaction with the PetC1 protein as outlined above. The presence of PetC1 degradation products could be excluded because (a) the PetC1 antibody failed to detect a protein with a molecular mass of 14 kDa, and (b) using membranes from the *Synecho- cystis* ΔpetC3 mutant\(^5\) no cross-reaction with a 14-kDa protein could be detected with the anti-PetC3-antibody (Fig. 7).

By using the anti-PetC2 antibody, no gene product could be detected in the isolated membranes of *Synechocystis*. To determine whether PetC1 and PetC3 are integral subunits of the cytochrome $b_{c2}$ complex, immunoblots were done with the isolated cytochrome $b_{c2}$ complex from *Synechocystis* (Fig. 7). From these blots it is evident that the two different Rieske proteins are not only associated with *Synechocystis* membranes, but also with the isolated cytochrome $b_{c2}$ complex. When using an identical protein concentration for the immunoblot analysis the cross-reaction of the anti-PetC1 antibody appeared much faster than with the anti-PetC3 antibody. Because both peptide antibodies show a similar cross-reactivity with the overexpressed Rieske proteins, this result indicates a much lower content of PetC3 in our cytochrome $b_{c2}$ complex preparation than of PetC1. A this supported by the fact that the isolated cytochrome $b_{c2}$ complex showed, even in a silver-stained polyacrylalide gel, no band at 14 kDa corresponding to PetC3, whereas all other subunits are present in a ratio of about 1:1 (Fig. 8).

**DISCUSSION**

**Heterologous Overexpression of Functional Rieske Proteins**—Although *E. coli* is the most popular host for the heterologous overexpression of foreign proteins, the overexpression of membrane proteins is often toxic and can have lethal consequences (8). There are, however, several examples in which successful overexpression of functional membrane proteins in *E. coli* cell membranes has been achieved. Specifically, the functional overexpression of Rieske proteins from hyperthermophilic organisms with incorporated iron-sulfur cluster has been reported (11, 12).

To our knowledge the overexpression of full-length Rieske proteins with incorporated iron-sulfur cluster from a mesophilic

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\(^3\) S. Anemüller, unpublished observations.

\(^4\) The abbreviation used is: WT, wild type.
organism in *E. coli* has never been reported. Here we show the overexpression of PetC proteins from mesophilic organisms in their native form with incorporated iron-sulfur cluster. The fact that all three overexpressed PetC proteins were localized in inclusion bodies or cell membranes of *E. coli* but not in the soluble cell fraction argues for the existence of a membrane anchor in these proteins similar to other Rieske proteins (4).

**Three Rieske Proteins Are Encoded in the Genome of Synechocystis**—The three overexpressed proteins were characterized by EPR measurements and typified as Rieske proteins by the presence of the Rieske [2Fe-2S] cluster \( g_s = 1.90 \) value. Although the \( g \) values of PetC1 and PetC2 were almost identical, the EPR spectrum of PetC3 differs slightly. The \( g_s \) value is shifted to a higher magnetic field, and the \( g_x \) value, which was not detectable for the other two Rieske proteins, could be easily recognized.

The midpoint potential determined by EPR-monitored redox titration for PetC1 (+320 mV) is typical for cytochrome *bc* complex Rieske proteins from ubi- and plastoquinol-containing organisms that show a highly positive midpoint potential of around +300 mV (12). In contrast, proteins with a low midpoint potential of about +135 mV, as determined for PetC3, are not found in the cytochrome *bc* complexes of any of these organisms.

**petC3 Encodes an Unusual Rieske Protein**—The sequence alignment of the three *Synechocystis* Rieske proteins in Fig. 1 clearly showed the two cluster binding domains in petC3, which are typical for Rieske proteins. Although the so-called Box II is formed by the CPCHGSXY motif typical of plasto- or ubiquinol-containing organisms, the sequence of PetC3 shows amino acid exchanges resulting in the sequence CPCHGAFX. The replacement of Ser\(^6\) of the binding motif as well as the Tyr\(^8\) → Phe substitutions can also be found in some other organisms (12), with both single amino acid exchanges resulting in a lowered midpoint potential of the expressed protein. In *Sacharomyces cerevisiae* the Ser\(^6\) → Ala mutation lowered the midpoint potential of the protein from +285 to +155 mV and the mutation Tyr\(^8\) → Phe from +285 to +217 mV (7). After double substitution, the expressed protein showed a low \( E_m \) value of +105 mV, and the resulting sequence of the Box II was CPCHGZX as in the sequence of the PetC3 protein. The midpoint potential of the mutated protein from *S. cerevisiae* correlates well with the determined \( E_m = +135 \) mV of PetC3. In conclusion, besides the exact determination of the \( E_m \) by spectroscopic methods, a low midpoint potential of PetC3 could be predicted by comparison with other PetC sequences and the characteristics of the encoded Rieske proteins. *Heliobacillus mobilis* also contains a
Rieske protein with a Box II sequence identical to the PetC3 protein (24) and with a similar midpoint potential of +150 mV. Interestingly, all organisms with the indicated single amino acid exchanges as well as organisms with a double mutation like H. mobilis use menaquinol instead of ubiquinol as electron donor for their cytochrome bc complex (12).

The fact that the amino acid sequence of the two boxes ligating the iron-sulfur cluster is identical in PetC1 and PetC2 argues for a midpoint potential of about +300 mV for the PetC2 protein, which is in good agreement with the observed completely reduced PetC1 and PetC2 proteins under aerobic conditions and in contrast with the oxidized PetC3.

On the Presence and Function of Three Rieske Proteins—Immunoblotting analysis clearly showed the presence of at least two petC gene products, PetC1 and PetC3, associated with membranes of the cyanobacterium Synechocystis PCC6803. The presence of the two proteins is the first evidence for the functional expression of various Rieske proteins in cyanobacteria. Although the functional expression of PetC2 was not indicated by immunoblot analysis, preliminary results with a promoter probing vector showed that PetC2 is also expressed to a certain degree in Synechocystis. ² This suggests that the anti-PetC2 antibody used in the immunoblot analysis showed an insufficient cross-reactivity.

Our investigations on the isolated cytochrome bc₆f complex of Synechocystis clearly showed that at least two Rieske genes are continuously expressed, and the resulting subunits are associated with this complex in vivo, although apparently in different amounts. Consequently, the purified cytochrome bc₆f complex from Synechocystis PCC6803 is heterogeneous with respect to its Rieske subunit. As the gene petC1 is organized with petA in one operon, it was likely that the encoded protein is the predominant form in the complex.

Mutagenesis experiments suggested that the gene encoding PetC2 cannot be completely deleted if the expression of PetC1 is strongly diminished, whereas the petC2 gene can easily be deleted without a resulting phenotype in the WT strain. ¹ These results are in good agreement with the determined characteristics of the isolated proteins. Apparently, PetC1 and PetC2 not only show a high sequence homology but also display similar midpoint potentials, suggesting that PetC2 should be able to replace PetC1. In contrast, other deletion experiments suggested that PetC3 is not able to replace the main Rieske protein PetC1. This is in agreement with the unusual characteristics of PetC3 reported here, especially with the low midpoint potential, which may not allow this protein to act as a Rieske protein in a PQ-oxidizing cytochrome bc₆f complex. However, in contrast to the petC2 gene, the deletion of petC3 resulted in a phenotype indicating a physiological function of the encoded protein in Synechocystis. ¹

Role of the Midpoint Potential for the Cytochrome bc₆f Complex Activity—Up to now, no Rieske protein with a similar low midpoint redox potential as PetC3 has been reported for any cytochrome bc₆f complex. As shown in Ref. 7, the activity of the cytochrome bc complex of S. cerevisiae decreases with decreasing midpoint potential of the Rieske protein. Nevertheless, even cells expressing mutant Rieske proteins with a single substitution (Ser⁶ or Tyr⁶), which lowered the midpoint potential to about +200 mV, were able to grow on ethanol/glycerol medium, although with a 30–40% lowered cytochrome bc₃ complex activity. These results argue for the general compatibility of this single amino acid exchange with ubiquinol as the electron donor for the Rieske protein. In contrast, a mutant strain with an Ser⁶ → Ala exchange in the Rieske protein resulting in an $E_m = +155$ mV was unable to grow on ethanol/glycerol medium, i.e. a Rieske protein with such a low $E_m$ value seems not to be able to oxidize ubiquinol efficiently. Because the $E_m$ values of ubi- and plastoquinol are similar (25), it seems appropriate to assume similar data as those obtained with a bc₃ complex-containing organism also for the plastoquinol-oxidizing cytochrome bc₆f complex. Results obtained from the mutagenic studies with the S. cerevisiae Rieske protein argue against a possible role of PetC3 in a plastoquinol-oxidizing reaction. However, some bacteria have been reported to contain cytochrome bc complexes with Rieske proteins of low redox potentials, i.e. between +100 and +200 mV; these were found to use menaquinol with a low midpoint potential of about −60 mV instead of plastoquinol/ubiquinol as electron carrier (26). From this point of view and considering the fact that PetC3 has been identified in this report in highly purified cytochrome bc₆f complexes, a conceivable function of PetC3 seems to be part of a cytochrome bc₆f complex that oxidizes menaquinol instead of plastoquinol. Although the metabolism for naphthoquinol biosynthesis has already been investigated for Synechocystis and all enzymes involved have been identified (27), nothing is known yet about free naphthoquinone in this cyanobacterium. The present work suggests the existence of a naphthoquinol-oxidizing cytochrome bc₆f complex; its location and possible physiological function remains to be determined in future experiments.

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