The Peroxide Complex of Yeast Cytochrome c Peroxidase Contains Two Distinct Radical Species, Neither of Which Resides at Methionine 172 or Tryptophan 51*

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The nature of the free radical species observed in the peroxide complex of yeast cytochrome c peroxidase is described for protein variants containing amino acid substitutions at Met-172 and Trp-51. As was the case with Met-172 mutations (Goodin, D. B., Mauk, A. G., and Smith, M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1295-1299), Trp-51 can be substituted to give active enzyme. Phe-51-containing enzyme has a higher turnover rate than the original enzyme and exhibits an altered pH dependence. The properties of the isotropic and axial components (Hoffman, B. M., Roberts, J. E., Kang, C. H., and Margoliash, E. (1981) J. Biol. Chem. 256, 6556-6564; Hori, H., and Yonetani, T. (1985) J. Biol. Chem. 260, 349-355) of the EPR signal of the wild-type enzyme-peroxide complex, studied as a function of $H_2O_2$ stoichiometry, support proposals (Goodin et al. (1985) and Hori and Yonetani (1985), see above) that two distinct radical species are formed, and spin quantification shows that the isotropic radical is always formed in substoichiometric amounts. The peroxide complexes for proteins containing amino acid substitutions at either Met-172 or Trp-51 exhibit somewhat larger than normal levels of the isotropic radical signal. In addition, these mutants are unlike wild-type enzyme in that the axial EPR signal associated with the peroxide complex is seen only at 10 K and not at 90 K. Thus, neither amino acid can be considered to be the molecular species responsible for either radical signal, but both mutations appear to affect the physical properties of the axial signal representing the major radical species.

Among the many metalloenzymes that are known, yeast cytochrome c peroxidase (ferrocyanochrome-chydrogen-peroxide oxidoreductase, EC 1.11.1.5) is unusual in that it and an unrelated ribonucleotide reductase (4) are the only two peroxidases, notably horseradish peroxidase compound I, that retains the second oxidizing equivalent on the porphyrin macrocycle as a π cation radical (6). The ES complex of cytochrome c peroxidase contains a unique free radical EPR signal that has long been ascribed to a reversibly oxidizable amino acid residue (7-9). This free radical is magnetically isolated from the heme iron (9), and its properties are thus inconsistent with a porphyrin-based radical (2). Two potentially oxidizable residues, Trp-51 and Met-172, have been identified based on their close positioning relative to the heme center (10, 11) and the physical properties of the radicals they might produce (2, 7, 9, 12-14).

The EPR properties of the ES radical have been well studied (2, 3, 7-9, 14). At temperatures below 25 K, the observed radical appears as an axial signal with $g_1 = 2.006$ and $g_1 = 2.034$ (2, 3) which is difficult to saturate, indicating an efficient spin relaxation mechanism. It has been argued that the properties of this signal are inconsistent with a radical of an aromatic residue but are possibly attributable to the intrinsic anisotropy of a nucleophilically stabilized sulfur radical (2, 9). At temperatures above 25 K, this axial signal is greatly broadened, presumably by spin lattice relaxation, so that only broad wings around $g = 2$ are observed (3). Superimposed on this broad resonance is an isotropic signal at $g = 2.004$ containing hyperfine structure that is easily saturated and is orientation-independent. These properties led Hori and Yonetani (3) to propose that ES contains more than one distinct radical species.

In a previous study, we have replaced Met-172 of cytochrome c peroxidase by site-directed mutagenesis to examine its role in the ES complex (1). It was found that Met-172 could be replaced to give active enzyme. EPR spectra at 90 K of the ES complex of cytochrome c peroxidase containing Ser-172 showed the presence of the isotropic signal, but lacked the broad wings. These results were consistent with a heterogeneity of radical species, but it was not possible to determine definitively if the axial signal was abolished by replacement of Met-172 or otherwise altered in its properties so that it was no longer observed at 90 K. In this study, we present additional evidence for two distinct radical species in ES, assess the relative contribution made by the isotropic signal, and
further investigate the EPR properties at 10 K for cytochrome c peroxidase containing mutations at Met-172 or Trp-51.

MATERIALS AND METHODS

Construction and Expression of Mutant Cytochrome c Peroxidase—The site-directed mutagenesis methods of Zoller and Smith (15) were used as previously described (1) for the construction of cytochrome c peroxidase mutants in the vector pEMBLCCP1 with the following exceptions. The mutation efficiency was increased significantly by adapting methods of Kunkel (16) for incorporation of uracil into the single-stranded template DNA. Briefly, 10 ml of an overnight culture (grown ampicillin) of the dut- ung* Escherichia coli RI0132 (17) freshly transformed with pEMBLCCP1 was diluted into 20 ml of the growth medium and incubated for 1 h at 37 °C. No additions of uridine to the growth medium were made. The culture was superinfected at a multiplicity of infection of 40 with f(1R1) bacteriophage followed by incubation at 37 °C for 4-6 h before isolation of single-stranded DNA as earlier described (18). Template DNA containing uracil was checked for a high differential survivability in ung* and ung* E. coli strains as described by Kunkel (16), and efficiencies were typically 10-fold higher in RI0132 than in JM101 (19). In the mutagenesis reactions, ratios of the mutagenic oligonucleotide to primer were reduced from 20:1 as described by Zoller and Smith (15) to 2:1 or 1:1, and the second primer was omitted. Mutagenesis efficiencies of 60-80% were consistently obtained, and this enabled the construction of families of mutations at a given amino acid by transformation of JM101 with as much as 0.25 pmol of DNA from the mutagenesis reaction, plasmid DNAs from ampicillin-resistant mutants which contained the following base mixtures for the Trp-51 gene) contained on the 1.7-kilobase HindIII fragment of pEMBLCCP1 with the following exceptions. The mutation efficiency was increased significantly by adapting methods of Kunkel (16) for incorporation of uracil into the single-stranded template DNA. Briefly, 10 ml of an overnight culture (grown ampicillin) of the dut- ung* Escherichia coli RI0132 (17) freshly transformed with pEMBLCCP1 was diluted into 20 ml of the growth medium and incubated for 1 h at 37 °C. No additions of uridine to the growth medium were made. The culture was superinfected at a multiplicity of infection of 40 with f(1R1) bacteriophage followed by incubation at 37 °C for 4-6 h before isolation of single-stranded DNA as earlier described (18). Template DNA containing uracil was checked for a high differential survivability in ung* and ung* E. coli strains as described by Kunkel (16), and efficiencies were typically 10-fold higher in RI0132 than in JM101 (19). In the mutagenesis reactions, ratios of the mutagenic oligonucleotide to primer were reduced from 20:1 as described by Zoller and Smith (15) to 2:1 or 1:1, and the second primer was omitted. Mutagenesis efficiencies of 60-80% were consistently obtained, and this enabled the construction of families of mutations at a given amino acid by transformation of JM101 with as much as 0.25 pmol of DNA from the mutagenesis reaction, plasmid DNAs from ampicillin-resistant colonies were sequenced (20) to screen for desired amino acid replacements.

Protein Expression and Purification—Using methods described earlier (1), mutants of the CCP gene (the cytochrome c peroxidase gene) contained on the 1.7-kilobase HindIII fragment of pEMBLCCP1 were subcloned into the yeast vector YEp13CCP which was used to transform yeast strains DG15-4 (a, leu2, his3, CCP::HIS3) or DG15-10 (a, leu2, his3, ura3, CCP::HIS3, PEP4::URA3). These yeast strains were constructed by crossing ZA515 (a, bar1, leu2, PEP4::URA3) with a strain W303-1AX (a, ura3, his3, CCP::HIS3) which was in turn derived from W303-1A A2H used previously (1). The pep4 phenotype was verified using the method of Jones (21). Mutant CCP genes were recovered from transformed yeast cultures and sequenced to verify the mutations. Yeast whole cell lysates were prepared for activity analysis and Western blot as earlier described (1).

Purification of cytochrome c peroxidase from 40-liter cultures of transformed yeast was performed by published methods (1, 22) with the following exceptions. Some mutants of cytochrome c peroxidase appear to be unstable during cell autolysis in ethyl acetate. For this reason, yeast cells were broken by mechanical fracture. Approximately, 1 kg of air-dried yeast was suspended in enough buffer A (consisting of 50 mM sodium acetate (pH 5.0), 1 mM EDTA, 1 mM diethiothreitol, and 1 mM phenylmethylsulfonyl fluoride) to make the suspension pourable. The mixture was frozen by pouring into liquid nitrogen and fractured by grinding three times for 1 min in a stainless steel blender. The freezing/grinding step was repeated once, and 3 liters of buffer A were added. After thawing and centrifugation at 10,000 g for 20 min, the clear supernatant was loaded onto a 5 × 15-cm DEAE-Sepharose CL-6B column equilibrated in 50 mM sodium acetate (pH 5.0). From this point, the protein purification followed the method previously published (1, 22).

Functional and Spectroscopic Measurements—Steady-state kineticsof the cytochrome c peroxidase-catalyzed oxidation of horse heart cytochrome c by H2O2 rates were performed as described by Kang and Erman (23) and were carried out at 25 °C in 20 mM Tris HCl buffer, 113 μM H2O2, >95% reduced cytochrome c at 20 μM, and approximately 300 pmol cytochrome c peroxidase. EPR spectra at 90 K were collected at 9.18 GHz using 1-mW microwave power, 20-G field modulation, and a receiver gain of 2.5 × 104. EPR spectra collected in Fig. 1 are the microwave power dependence of the 90 K EPR signal intensity observed at the magnetic field position of the maximum in the isotropic signal at g = 2.004. A different dependence was observed when the H2O2 to cytochrome c peroxidase ratio was raised from 0.25 to 2.5. This observation strongly supports the notion that a mixture of radical species exists in which a greater contribution of the more easily...
power at two ratios of concentration of 100 oxidase 30% glycerol. Two curves are shown: for $[\text{H}_2\text{O}_2]/[\text{cytochrome c peroxidase}] = 0.25 (\bullet)$ and for $[\text{H}_2\text{O}_2]/[\text{cytochrome c peroxidase}] = 2.5 (O)$. 

**Table I**

| EPR spin quantification of the isotropic component of the ES EPR signal at 90 K |
|---------------------------------|
| Spectra were collected at 6-$\mu$W microwave power and 10-G field modulation. The spin concentration $([S])$ was determined by double integration using 152 $\mu$M $\text{K}_2\text{NO(SO}_2\text{)}_2$ as a spin standard. |

| [CCP]$^*$ | $[\text{H}_2\text{O}_2]$ | $[\text{H}_2\text{O}_2]/[\text{CCP}]$ | $[S]$ | $[S]/[\text{CCP}]$ |
|-----------|----------------|----------------|------|--------------------|
| WT CCP    | 200            | 2000           | 10.0 | 16.3               | 0.682 |
| WT CCP    | 200            | 200            | 1.0  | 9.6                | 0.048 |
| CCP(F51)  | 121            | 188            | 1.6  | 12.0               | 0.099 |

$^*$ CCP, cytochrome c peroxidase.

**Fig. 2.** Microwave power dependence of EPR signal intensity at two ratios of $[\text{H}_2\text{O}_2]$ to [cytochrome c peroxidase]. EPR signal intensity ($S$) was measured at 90 K as the peak-to-peak amplitude of the isotropic signal at 3240 G as a function of microwave power ($P$). Both samples contained cytochrome c peroxidase at a concentration of 100 $\mu$M in 100 mM sodium phosphate (pH 6.0) and 30% glycerol. Two curves are shown: for $[\text{H}_2\text{O}_2]/[\text{cytochrome c peroxidase}] = 0.25 (\bullet)$ and for $[\text{H}_2\text{O}_2]/[\text{cytochrome c peroxidase}] = 2.5 (O)$.

**Fig. 3.** Relative cytochrome c peroxidase activities and Western blot of transformed yeast whole cell lysates. Yeast whole cell lysates were assayed for cytochrome c peroxidase activities as earlier described (1). For Western blotting, 5 $\mu$l of each cell lysate, corresponding to 50 $\mu$l of cell culture, was run on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. After transfer to nitrocellulose and blocking (37), the filter was probed using rabbit anti-cytochrome c peroxidase serum and detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (38). Sample 1 was from yeast strain DG5-4 transformed with YEp13 containing no cytochrome c peroxidase sequence (39), samples 2-9 were from yeast strain DG5-4 transformed with YEp13CCP containing either wild-type cytochrome c peroxidase gene sequence or the specified mutation.

Strains were derived from the earlier described host W303-1A A2H, and DG5-10 contained a URA3 interruption of PEP4, resulting in vacuolar protease deficiency (25). These variations in protein level and total activity may thus represent differences in the intrinsic enzyme stability, in the mitochondrial membrane transport, or in heme binding properties of the precursor apoprotein. Nevertheless, these observations demonstrate that a number of different amino acids can replace Trp-51 to give cytochrome c peroxidase with catalytic activity.

Samples of cytochrome c peroxidase containing Phe-51 (CCP(F51)) and Trp-51 (WT CCP) were purified as earlier described (1). The optical spectrum of CCP(F51) gave an $A_{408}/A_{340}$ ratio of 1.38. This was slightly higher than 1.32 observed for WT CCP but was otherwise similar to that of WT CCP. Addition of $\text{H}_2\text{O}_2$ to CCP(F51) produced an absorbance spectrum that was characteristic of the ES complex (26) (data not shown) but with somewhat broadened transitions at 519 and 548 nm relative to those of WT ES. These properties are in agreement with those observed by Fishel et al. (27) for cytochrome c peroxidase containing Phe-51 produced in *E. coli*.

The purified protein containing Phe-51 exhibited an initial turnover rate at pH 5.6 with horse heart cytochrome c that was approximately 5 times higher than the Trp-51-containing...
enzyme, as also observed by Fishel et al. (27). Shown in Fig.
4 is the pH dependence of the initial turnover rate in 100 mM
phosphate buffer. In addition to the overall increased turnover
rate, CCP(F51) exhibited a markedly different pH dependence
in which the maximal rate was observed at pH 6.5 rather than
5.0 as observed for WT CCP and CCP(S172).

EPR spectra of the native ferric forms of WT CCP,
CCP(C172), and CCP(F51) at 10 K are shown for the g = 6
region in Fig. 5. These spectra are roughly similar in the
position of their turning points. EPR spectra (not shown) for
CCP(C172) are essentially identical to that shown for
CCP(C172). The apparent broadening of the component of
the signal at g = 6.65 (980 G) in the spectrum of WT CCP of
Fig. 5 was not always observed and is somewhat variable from
preparation to preparation. However, for CCP(F51), an addi-
tional set of turning points is apparent as two shoulders on
either side of the g = 6 transition.

**EPR Properties of Mutant Cytochrome c Peroxidase-Perox-
dase Complexes**—EPR spectra at 90 K for WT CCP,
CCP(F51), and their ES complexes are shown in Fig. 6. The
ES complexes were generated by the addition of a slight excess
of H₂O₂. As clearly seen in the g = 2 region, the ES complex
of CCP(F51) contained the narrow isotropic component of
the ES radical signal. The hyperfine structure observed for
this signal under low field modulation conditions (23) was also
present. Potentially more significant are the differences ob-
erved in the wings of the free radical EPR signals of Fig. 6.
The axial EPR signal associated with the ES complex, ob-
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From the relative gain settings of Fig. 6, it is apparent that
the amplitude of the narrow component of the signal is greater
for CCP(F51) than observed in the sample of WT CCP. Spin
quantification of this isotropic signal for CCP(F51) ES (Table
1) showed that it was nevertheless present at less than 10%
of the total cytochrome c peroxidase concentration. This
conclusion was verified by double integration of the two
spectra of Fig. 6. The spin concentration of the isotropic
signal observed for CCP(F51) ES at 90 K represented only
17% of that obtained by integrating the spectrum of WT  ES
complex. The relative receiver gains are displayed at the left of each scan.

![FIG. 6. EPR spectra at 90 K for WT CCP and CCP(F51) and
their peroxide complexes.](image)

**Fig. 6. EPR spectra at 90 K for WT CCP and CCP(F51) and
their peroxide complexes.** Spectra were collected using 50-mW
microwave power and 10-G field modulation for 121 μM cytochrome
c peroxidase in 100 mM sodium phosphate (pH 6.0) and 50% glycerol
and a small excess of H₂O₂. Sample A, WT CCP; sample B, WT ES
complex; sample C, CCP(F51); sample D, CCP(F51) ES complex. The
relative receiver gains are displayed at the left of each scan.

![FIG. 4. Initial enzyme turnover rate as function of pH for WT CCP, CCP(S172), and CCP(F51).](image)

**Fig. 4. Initial enzyme turnover rate as function of pH for WT CCP, CCP(S172), and CCP(F51).** Enzyme kinetics were performed as described by Kang and Erman (23) using conditions described under "Material and Methods." Samples were: WT CCP ( ), CCP(S172) ( ), and CCP(F51) ( ) (right scale).

![FIG. 5. EPR spectra at 10 K for ferric cytochrome c perox-
dase samples.](image)

**Fig. 5. EPR spectra at 10 K for ferric cytochrome c perox-
dase samples.** Spectra were collected for 100 μM cytochrome c
peroxidase in 100 mM sodium phosphate (pH 6.0) using 1-mW micro-
wave power and 2-G field modulation. Sample A, WT CCP; sample
B, CCP(C172); sample C, CCP(F51).
observed at 90 K. Significant errors may be introduced in such calculations (28) arising from small base-line changes or from a significant contribution from the axial wings if they extend well beyond 1500 G from the signal center.

EPR spectra at 10 K of the ES complexes generated from samples of WT CCP, CCP(S172), CCP(C172), and CCP(F51) are shown in Fig. 7. Each of these spectra shows the presence of varying amounts of the axial EPR signal of the ES radical. For some samples, addition of exact stoichiometric amounts of H2O2 appeared to result in the incomplete formation of the ES complex, as judged by residual levels of the g = 6 ferric EPR signal; and these samples had smaller than normal amplitudes of the axial ES EPR signal. However, by addition of a slight excess of H2O2 (1.5-fold) followed by rapid (15 s) freezing, samples of CCP(F51) ES were prepared that had essentially the same signal intensity as observed for equimolar WT ES. It has been noted that the ES complex for cytochrome c peroxidase containing Phe-51 is less stable than the wild-type complex (27), and this may explain the observed variability in these samples. Nevertheless, the EPR results of Fig. 7 clearly show that the axial signal species can be observed at 10 K for the ES complex containing amino acid replacements for either Met-172 or Trp-51.

DISCUSSION

The data presented above, aided by the study of forms of the enzyme altered by site-directed mutagenesis, allow a number of conclusions to be drawn concerning cytochrome c peroxidase and its peroxide complex. Neither Met-172 or Trp-51 is necessary for cytochrome c peroxidase to function as a peroxidase. However, substitution of Trp-51 produces observable effects on the initial rate (Fig. 4 and Ref. 27) and pH dependence of its kinetics as well as a small structural perturbation in the symmetry of the ferric heme EPR signal (Fig. 5). The axial and isotropic EPR signals of the ES complex are observed to be formed independently, and the axial signal represents the major species. Neither of these signals can be attributed to either Met-172 or Trp-51, although replacement of either residue appears to alter the properties of the axial signal so that it is difficult to observe at 90 K.

The alteration in the magnitude of the steady-state rate and its pH dependence observed upon substitution of Trp-51 are not fully understood. Although it has been shown that a group with a pK = 5.5, possibly His-52, must be unprotonated for the efficient reaction of native cytochrome c peroxidase with H2O2 (29), the changes observed in these overall steady-state rates may include other factors such as binding and release of cytochrome c and intermolecular as well as intramolecular electron transfer steps. Thus, these reaction components must be studied individually to assess which are responsible for the changes observed upon substitution of Trp-51. These studies are in progress. The observed kinetics, however, indicate that the rate-limiting step in the mechanism has been altered by the substitution. If the kinetic model that has been proposed (23) is correct, the rate-limiting step involves electron transfer within the cytochrome c peroxidase-cytochrome c complex rather than cytochrome c binding or reaction of cytochrome c peroxidase with H2O2.

The EPR properties of the native enzyme (Fig. 5) indicate that CCP(F51) contains an additional conformational substate that can be distinguished by a slightly different symmetry of the heme center. The apparent anisotropy of this signal in cytochrome c peroxidase has been earlier described (8) as arising from the contribution at pH 6 of two different forms of the iron center having slightly different symmetries. One component is represented by the central line at g = 6.0 arising from high spin ferric iron in a purely axial environment. This is superimposed on a smaller signal containing two turning points flanking the g = 6 signal at g = 5.18 and 6.65 (980 and 1260 G) and represents a distinct form of axial iron with a small rhombic distortion. Presumably, cytochrome c peroxidase exists in solution as a mixture of two conformations which result in small symmetry differences in the heme environment. The present data indicate that CCP(F51) exists as a mixture not only of the axial and rhombic forms present for WT CCP, CCP(S172), and CCP(C172), but contains an additional minority conformation with an intermediate degree of rhombic distortion. Trp-51 is hydrogen-bonded to a water molecule that resides 2.4 Å above the distal heme iron (10).

Although this water molecule has been described as ligating to the iron atom with a slightly longer than normal distance (10), recent reports have shown that resonance Raman spectra of ferric cytochrome c peroxidase at pH 6 are more characteristic of a five-coordinate than a six-coordinate heme protein (30, 31). It is clear that substitution of Trp-51 by Phe-51 will cause a change in the hydrogen bonding of this water, and its effects may be subtly reflected in the anisotropy of the ferric EPR signals.

From the EPR data on the ES complex, we conclude that the narrow isotropic and broad axial EPR signals observed for the ES complex represent distinct species as earlier proposed (1, 3), that the isotropic signal species is formed independently of the axial species, and that it usually represents only a small fraction of the total number of spins associated with the ES complex. A similar signal has been observed in substoichiometric levels in samples of myoglobin oxidized with H2O2 (32). It is therefore likely that under physiological conditions, the isotropic species does not figure prominently in the composition or function of the ES complex. It may represent a residue oxidized by H2O2 in a side reaction or result from the spontaneous decomposition of ES. The latter interpretation is not likely, however, as we have observed that
in samples of WT ES stored at room temperature for approxi-
ately 15–20 min, there is no increase in the ratio of the
isotropic to axial signal components. It is also possible that
the same molecular species gives rise to both signal compo-
nents. This could occur if two conformations of the species
existed that would result in the radical having different prop-
erties. Indeed, the existence of two conformational forms of
the radical has been proposed to explain properties of the
equilibrium intramolecular distribution of oxidizing equiva-

cents in ES (33, 34). However, it would be easy to
imagine two radical conformations having different spin
relaxation properties, it would be more difficult to explain the
greatly different anisotropies unless these properties were
determined by spin–spin dipolar interaction with the ferriyl
heme center. Arguments have been previously presented (2)
that such an interaction would not be large enough to produce
the observed anisotropy.

The presence of the EPR signals of the ES complex in
samples of cytochrome c peroxidase altered by site-directed
mutagenesis demonstrates that neither Met-172 or Trp-51
contains the molecular species of either of the observed radicals. This conclusion is consistent with a magnetic circular
dichroism study in which no evidence for alteration of trypt-
ophan residues was observed in the ES complex (35). The
increased levels of the minority isotropic spin species observed
for CCP(F51) ES and CCP(S172) ES (1) may result from a
more rapid decay of an ES complex that is less stable than
that of WT CCP (27), in the increased accessibility of a
residue oxidized by H2O2 during a side reaction, or in the
presence in the mutant proteins of a different contribution from
distinct conformations of the radical site.

The absence of detectable axial EPR signals at 90 K for
the ES complexes of mutants at Met-172 or Trp-51 which are
nevertheless observed at 10 K must result from an alteration
of the radical site; however, the similar effects of
two different mutations, near to but on opposite sides of the
heme, suggest that the properties of the radical may be influ-
enced by the heme itself. Mössbauer studies (36) have shown
that the ferryl centers of heme proteins usually exist as an S
= 1 state with a large zero-field splitting of approximately 28 K
so that the magnetic states may not be significantly popu-
lated at low temperature. A weak interaction between the
ES radical species and the thermally excited states of this center
may provide the efficient and highly temperature-dependent
spin relaxation characteristic of the axial radical. The two
mutations near the heme may have simply altered the magni-

tude of the zero-field splitting of the ferryl center and, as a
result, the temperature dependence of the observed EPR
properties. The temperature dependence of the magnetic
properties of these mutant enzymes needs to be studied to
evaluate these effects.

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