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Preparation of Catalytically Active Cytochromes P-450 by Antigen Exchange on Monoclonal Antibody Based Immunoadsorbents†

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ABSTRACT: Catalytically active cytochromes P-450 have been prepared by monoclonal antibody (MAb) directed immunopurification using an antigen-exchange technique. Immunoaffinity-purified cytochromes P-450 that require denaturants for efficient desorption from the immunoaffinity matrix, although significantly lacking in catalytic activity, were found to retain epitopic structural integrity as probed by radioimmunoassay using MAbs to 3-methylcholanthrene and phenobarbital-induced rat liver cytochromes P-450. These denatured cytochromes P-450 were capable of displacing from the immunoaffinity matrices epitopically related cytochromes P-450 that retained aryl hydrocarbon hydroxylase and 7-ethoxycoumarin *O*-deethylase activities. Such epitope-specific exchange of denatured for native antigen on a solid-phase matrix containing a MAb may be generally applicable to preparation of proteins with the retention of activity.

The cytochromes P-450 metabolize a variety of xenobiotic and endogenous compounds, including drugs, carcinogens, and steroids (Coon et al., 1980; Gelboin, 1980; Lu & West, 1980; Sato & Kato, 1982). The multiplicity of cytochrome P-450, which has been demonstrated by a variety of biochemical and immunological methods (Lu & West, 1980), is responsible for its broad spectrum of substrate specificity and reactivity. The metabolic fate of cytochrome P-450 substrates therefore depends on the type and amount of the cytochromes P-450 that are present. Progress in distinguishing closely related isozymes has been limited, however, by the difficulties encountered in purification of the individual isozymes and their overlapping enzymatic specificities.

As an approach to the multiplicity problem, we have prepared and characterized several panels of monoclonal antibodies (MAbs)[1] to different cytochromes P-450 (Park et al., 1980, 1982a,b, 1984). MAbs target specific epitopes on the antigen (Yelton & Scharff, 1981) and when coupled with

† This work is dedicated to the memory of Prof. Sherman Beychok.

[1] Abbreviations: MAb, monoclonal antibody; RIA, radioimmunoassay; MC, 3-methylcholanthrene; PB, phenobarbital; MC-P-450, major form of MC-induced rat liver cytochrome P-450; PB-P-450, major form of PB-induced rat liver cytochrome P-450; glycine-eluted P-450, cytochrome P-450 polypeptide eluted from immunoadsorbent with 0.1 M glycine (pH 3.0); PBS, phosphate-buffered saline (5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 150 mM NaCl, pH 7.4); AHH, aryl hydrocarbon hydroxylase; ECD, 7-ethoxycoumarin *O*-deethylase; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kDa, kilodalton; BCA, bicinchoninic acid.

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conventional characterization methods are precise probes for those cytochromes P-450 with the MAb-specific epitope. MAbs that inhibit cytochrome P-450 dependent catalytic activity have helped identify the cytochromes P-450 responsible for drug- and carcinogen-metabolizing activities in tissues from animals (Fujino et al., 1984a) as well as humans (Fujino et al., 1982, 1984b). In additon, MAb-directed radioimmunoassay (RIA) has detected and identified MAb-specific cytochromes P-450 in a variety of tissues and species (Cheng et al., 1984a; Song et al., 1984, 1985), and MAbs coupled to solid-phase supports have been used to successfully immunoadsorb and purify MAb-specific cytochromes P-450 (Lewis et al., 1981; Friedman et al., 1983; Cheng et al., 1984a,b; Reubi et al., 1984).

Additional insight into cytochrome P-450 multiplicity and regulatory mechanisms would be provided upon purification and characterization of the catalytic activities of the repertoire of isozymes present in various tissues and species under assorted conditions, such as exposure to certain xenobiotics. Progress toward purifying additional isozymes has been hindered by the difficulties encountered with conventional purification procedures, which are often lengthy and which may not readily separate structurally similar isozymes. MAb-based immunopurification is a highly specific alternative to conventional methods in that cytochromes P-450 are purified on the basis of epitopic content. The MAb-specific cytochromes P-450 prepared in this manner are of sufficient purity for electrophoretic characterization (Lewis et al., 1981; Friedman et al., 1983; Cheng et al., 1984a,b; Reubi et al., 1984) and amino acid sequence analysis (Cheng et al., 1984b; Ozols et al., 1985). They are, however, usually desorbed from the immunoaffinity matrix with reagents that typically inactivate enzymes and are thus unsuitable for characterization of catalytic activity. We now report that catalytically active cytochromes P-450 can be eluted from the matrix by an antigen-exchange technique. The method entails displacement of active enzyme from the immunoaffinity matrix by competition with the purified enzyme, which, although denatured, nevertheless displays integrity of its MAb-specific epitope. We present results using MAbs to MC-P-450 and PB-P-450 to prepare catalytically active MAb-specific cytochromes P-450 from liver microsomes of MC- and PB-treated rats, respectively. Although applied to cytochrome P-450, this exchange method is general in nature and should find broader application in immunopurification of other proteins in an active state.

Experimental Procedures

Preparation of Monoclonal Antibodies. These were prepared from culture medium of hybridomas or mouse ascites fluid as described (Park et al., 1982b, 1984). MAbs were purified by precipitation with 2.0 M ammonium sulfate and DEAE-Affigel Blue (Bio-Rad) chromatography. The MAbs used were 1-7-1 and 1-31-2 to MC-P-450 (Park et al., 1982b) and 2-66-3 to PB-P-450 (Park et al., 1984).

Preparation of Microsomes. Adult male Sprague-Dawley rats were treated with PB (80 mg kg^{-1} day^{-1} for 4 days; in PBS) or MC (30 mg kg^{-1} day^{-1} for 3 days; in corn oil), and the corresponding liver microsomes were prepared by differential centrifugation. They were suspended in 10 mM Tris-HCl and 20% glycerol and stored at −70 °C.

Radioimmunoassay. ^{3}H-Labeled MAbs were prepared by reductive methylation with [^{3}H]$NaBH_4$ (Tack et al., 1980) to a specific activity of 2×10^6 cpm/μg. The solid-phase competitive RIA was carried out as described (Song et al., 1984) by measuring the inhibition of binding of radiolabeled MAb to antigen. The wells of microtiter plates (96 wells, polystyrene; Immunlon 1 from Dynatech) were precoated with liver microsomes from MC- or PB-treated rats (2 or 5 μg per well, respectively) by overnight incubation at 4 °C. After the nonspecific sites were washed and blocked with 3% bovine serum albumin in PBS and then additionally washed with PBS, a mixture of [^{3}H]MAb and the competing antigen was transferred to the wells and incubated for 3 h at room temperature. The wells were then washed 3 times with PBS, and the binding of [^{3}H]MAb to the wells was measured.

Immunopurification. The MAbs were coupled to CNBr-activated Sepharose 4B (Pharmacia) (10 mg of MAb/mL of resin) in 0.2 M sodium chloride and 0.2 M sodium citrate (pH 6.5) as described (Friedman et al., 1983). Batchwise purified of cytochrome P-450 was carried out essentially as described (Friedman et al., 1983; Cheng et al., 1984a). Liver microsomes were solubilized with 1% Emulgen 911 (Kao-Atlas) by stirring for 30 min at 4 °C. Two milliliters of solubilized microsomes (5 mg/mL) was then incubated per milliliter of Sepharose–MAb for 5 min. The matrix was then successively washed with 4 mL of the following solutions, each containing 25% glycerol and 0.1% Emulgen 911: twice with 40 mM potassium phosphate (pH 7.2); twice with 40 mM potassium phosphate and 1 M NaCl (pH 7.2); twice with 4 mM potassium phosphate (pH 7.2). Cytochrome P-450 was desorbed from the resin with 2 mL of 0.1 M glycine hydrochloride (pH 3.0) and was adjusted to pH 7.2 with 1 M Tris-HCl (pH 8.5); this material will be referred to as glycine-eluted P-450.

Antigen Exchange. For preparation of catalytically active cytochrome P-450, Sepharose–MAb (1 mg of MAb/mL of resin) was first incubated with solubilized microsomes and extensively washed as described above, omitting the final glycine-elution step. The cytochrome P-450 containing resin was then incubated with end-over mixing at 4 °C with the glycine-eluted P-450. The immunoadsorbent was centrifuged and the supernatant removed and assayed for catalytic activities.

As a control, anti-lysozyme MAb HyHel 9 (provided by Dr. S. Smith-Gill, NCI) coupled to Sepharose was used. This Sepharose–MAb did not bind to any microsomal proteins as detectable by SDS–PAGE. In addition, treatment of this resin with microsomes as described above did not yield a supernatant with measurable catalytic activity.

Other Procedures. Protein concentration was determined by the Pierce BCA assay (Pierce) using bovine serum albumin as standard. Assays of AHH and ECD activities were performed according to standard procedures (Nebert & Gelboin, 1967; Greenlee & Poland, 1978) using a previously described enzyme reconstitution system that includes reductase (Park et al., 1984).

Results and Discussion

Immunopurification of Cytochromes P-450. We have prepared immunochemically related rat hepatic cytochromes P-450 by a previously reported immunopurification procedure (Cheng et al., 1984a). A 57-kDa polypeptide was obtained from liver microsomes from MC-treated rats with MAb 1-31-2 while both 57- and 56-kDa polypeptides were obtained with MAb 1-7-1. Amino-terminal sequence analysis has shown that the 57- and 56-kDa polypeptides correspond to the P-450c and P-450d isozymes, respectively (Cheng et al., 1984b). When Sepharose-bound MAb 2-66-3 was used to immunopurify cytochrome P-450 from liver microsomes from PB-treated rats, a PB-inducible, 54-kDa species was obtained (Friedman et al., 1983).

Catalytic Activity of Glycine-Eluted Cytochromes P-450. Effective desorption of cytochromes P-450 from immunoad-

Table I: Aryl Hydrocarbon Hydroxylase and 7-Ethoxycoumarin *O*-Deethylase of Cytochromes P-450 Prepared by Immunopurification and Antigen Exchange[a]

enzyme source	MC microsomes purified with Sepharose–MAb 1-7-1	MC microsomes purified with Sepharose–MAb 1-31-2	PB microsomes purified with Sepharose–MAb 2-66-3
	Aryl Hydrocarbon Hydroxylase[b]		
(1) immunoadsorbed P-450[c]	533	293	255
(2) glycine-eluted P-450	0.6	0.4	0.1
(3) antigen-exchanged P-450	16	23	12
	7-Ethoxycoumarin *O*-Deethylase[b]		
(1) immunoadsorbed P-450[c]	2297	1885	830
(2) glycine-eluted P-450	0.4	0.3	0.9
(3) antigen-exchanged P-450	73	112	98

[a] Sepharose–MAb resin (0.05 mL) was incubated with 0.1 mL of solubilized microsomes as described under Experimental Procedures. The Sepharose–MAb–cytochrome P-450 complex was then incubated with 0.8 mL of glycine-eluted P-450 for 24 h at 4 °C and centrifuged, and the supernatant activities were measured. [b] Activity expressed as picomoles per minute in total reaction volume. [c] Values determined by difference between microsomal activity and unadsorbed activity in pooled washes of Sepharose–MAb.

sorbents requires rather stringent elution conditions as is generally the situation in immunoaffinity purification of proteins (Scouten, 1981). Several laboratories have used glacial acetic acid (Lewis et al., 1981), thiocyanate (Reik et al., 1982), or SDS (Reubi et al., 1984) for elution of cytochrome P-450 from immunoadsorbents. These agents are highly denaturing and have therefore presumably eluted catalytically inactive cytochromes P-450. The problem of obtaining active protein by such immunoaffinity methods applies not only to cytochrome P-450 but is a general one, since an MAb with a high affinity toward an antigen requires strong conditions for disruption of the MAb–epitope interaction.

In our laboratory we employ 0.1 M glycine (pH 3.0) for elution of cytochromes P-450. The glycine-eluted P-450s prepared by this procedure were found to exhibit relatively low, yet clearly measurable, levels of AHH and ECD activity (Table I, lines 2). While measurements of these residual cytochrome P-450 dependent activities may be useful in characterizing cytochromes P-450 immunopurified from a variety of sources, such an approach has the major disadvantages that (1) an activity may be undetectably low in some cases and (2) some activities may be more susceptible to inactivation by 0.1 M glycine (pH 3.0) than others, and the relative observed activities toward different substrates may therefore not truly represent those of the native cytochrome P-450. We therefore sought an approach to immunopurify these enzymes under mild, nondenaturing conditions. The objective was to obtain sufficient active enzyme for catalytic characterization.

Preparation of Catalytically Active Cytochrome P-450 by Antigen Exchange. We sought an effective technique for disrupting MAb binding to cytochrome P-450 without resorting to solvent conditions that greatly perturb protein structure. Several solutions to this problem may be envisioned. These include addition of effector molecules that alter the conformation of the antigen in such a manner as to decrease the strength of the interaction between the antibody-combining site and the epitope. This method has limited applicability, however, since specific conformational modulators of the antigen of interest may not be available or known. Another approach for disrupting an antibody–antigen interaction is by competitive displacement of antigen from antibody with a synthetic peptide whose sequence corresponds to that of the MAb-specific epitope. The difficulty of this approach is that the primary sequence of the epitope must first be determined and the peptide must then be synthesized in a relatively laborious and expensive procedure.

We desired the specificity inherent in using the epitopic peptide for eluting active cytochrome P-450 from the immunoadsorbent without the need to predetermine the primary sequence of the epitopes recognized by each of our MAbs. Previous studies have demonstrated that cytochrome P-450 while yet immobilized on a Sepharose–MAb matrix remains active both enzymatically (Lewis et al., 1981) and spectrally (Friedman et al., 1983). We thus sought to determine whether the MAb-specific epitopes on the glycine-eluted P-450s also retained sufficient structural intergrity to be capable of competing with and displacing the adsorbed cytochrome P-450 from Sepharose–MAb.

The glycine-eluted P-450s obtained with MAbs 1-7-1 and 1-31-2 and with MAb 2-66-3 were examined by competitive RIA with MC and PB microsomes, respectively. We previously demonstrated that this RIA procedure is specific for cytochrome P-450 with the MAb-specific epitope and that unrelated proteins do not compete for binding (Song et al., 1984). Competition was observed between each of these glycine-eluted P-450s and the corresponding microsomes from which they were purified (Figure 1). The MAb-specific epitopes on the denatured antigens thus maintain a sufficient degree of structural integrity for competition with the cytochromes P-450 present in microsomes, although one cannot determine from these data whether the affinity of MAb for glycine-eluted P-450 is the same or less than that of native, microsomal cytochrome P-450. This result therefore suggested the feasibility of using the glycine-eluted P-450 for displacement of active enzyme from the immunoadsorbent.

Preparation of active, immobilized cytochrome P-450 was accomplished by incubating Sepharose–MAb 1-7-1 with MC microsomes, followed by extensively washing with neutral buffers to remove nonadsorbed material. Glycine-eluted P-450, which had been previously prepared with MAb 1-7-1 was then incubated with the Sepharose–MAb 1-7-1–cytochrome P-450 complex. Exchange of the added glycine-eluted P-450 for bound, active cytochrome P-450 was detected by centrifugation of the resin at different times and analysis of the supernatants for AHH activity. The time course of antigen exchange is shown in Figure 2. AHH activity increased in the supernatant after a 1-h incubation and was maximal after an incubation of 24 h. Subsequent antigen exchange experiments were thereafter carried out for a 24-h incubation period.

The antigen exchange process was further examined by incubating varying amounts of glycine-eluted P-450 purified by MAb 1-7-1 with Sepharose–MAb 1-7-1 bound cytochrome P-450, followed by analysis of the supernatants for AHH activity. The displaced activity is shown in Figure 3 and may be seen to initially increase with increasing levels of glycine-P-450. The decline in specific AHH activity above 0.2 mg of added glycine-eluted P-450 may be attributed to dilution

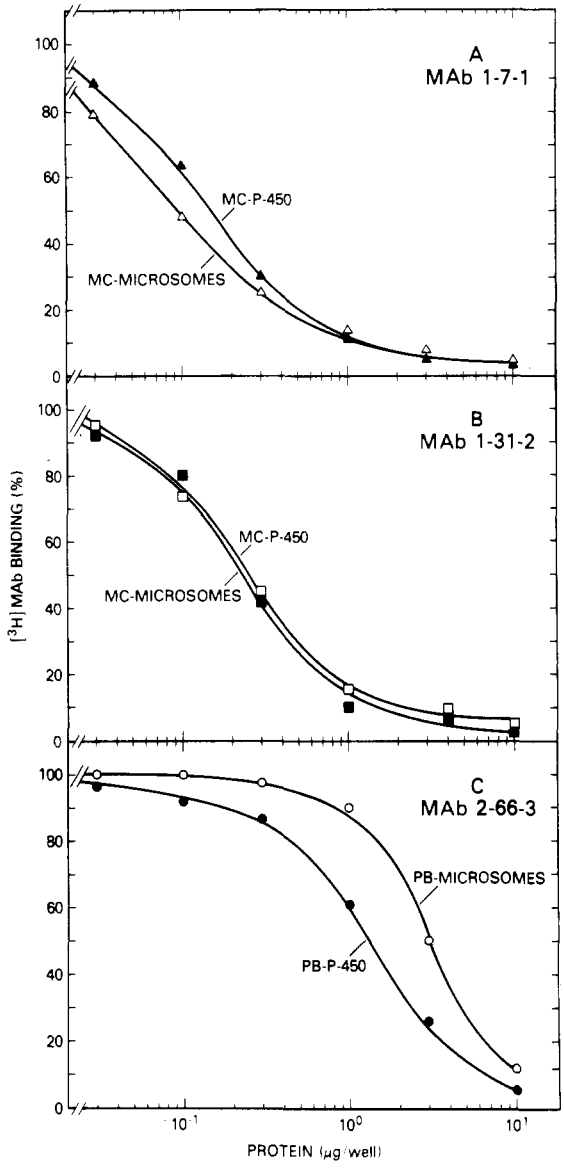

FIGURE 1: Radioimmunoassay of immunopurified, glycine-eluted cytochrome P-450. (A) Competitive RIA for MAb 1-7-1 immunopurified cytochrome P-450 (MC-P-450) using [^{3}H]MAb 1-7-1; (B) competitive RIA for MAb 1-31-2 immunopurified cytochrome P-450 (MC-P-450) using [^{3}H]MAb 1-31-2; (C) competitive RIA for MAb 2-66-3 immunopurified cytochrome P-450 (PB-P-450) using [^{3}H]MAb 2-66-3. Open and closed symbols represent MC or PB microsomes and immunopurified cytochromes P-450, respectively. Microtiter wells were precoated with 2 μg of rat liver MC microsomes (A and B) or 5 μg of PB microsomes (C), and 0.1 mL of each radiolabeled MAb (15000 cpm) was incubated overnight at 4 °C with the cytochromes P-450 immunopurified with the corresponding Sepharose–MAb. This mixture was then added to the microsome-coated microtiter wells and incubated at room temperature for 3 h. After washing the wells to remove unbound [^{3}H]MAb, the radioactivity bound to the wells was measured. Binding is expressed as percent of that in the absence of competing antigen. The 100% binding level was 600–800 cpm per well.

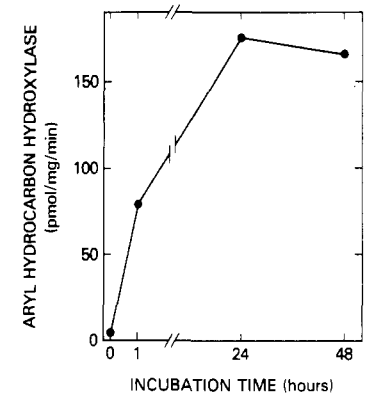

FIGURE 2: Time course of antigen exchange of cytochrome P-450. A total of 0.3 mL of the Sepharose–MAb 1-7-1–cytochrome P-450 complex was mixed at 4 °C with 0.6 mL (0.3 mg) of glycine-eluted P-450 prepared with MAb 1-7-1. Aliquots were incubated for different times and centrifuged, and the supernatant was assayed for AHH activity.

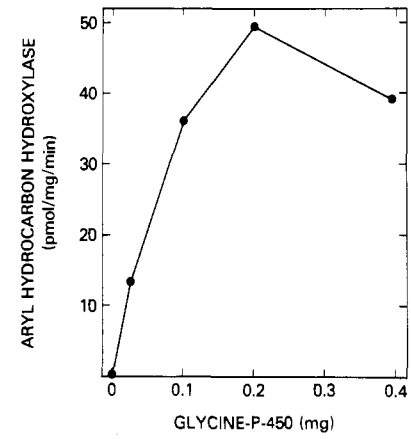

FIGURE 3: Effect of displacing antigen on antigen exchange of cytochrome P-450. A series of tubes containing 0.05 mL of Sepharose–MAb 1-7-1–cytochrome P-450 and 0.8 mL of varying amounts of glycine-eluted P-450 prepared with MAb 1-7-1 were mixed at 4 °C. After 24 h, the tubes were centrifuged and the supernatants assayed for AHH activity.

of displaced, active cytochrome P-450 with increasing amounts of added, relatively inactive glycine-eluted P-450. Increased AHH activity did not appear in the supernatant in the absence of glycine-eluted P-450, demonstrating that the observed AHH activity does not arise from mere dissociation of active cytochrome P-450 from the immunoafffinity matrix but rather results from a specific exchange process.

Antigen exchange was performed with the glycine-eluted P-450s purified with three MAbs (1-7-1, 1-31-2, and 2-66-3), and the active enzymes adsorbed to the corresponding Sepharose–MAb matrices. The AHH and ECD activities adsorbed to the resins (Table I, lines 1) represent a fraction of the total applied microsomal activities, since each Sepharose–MAb adsorbs from total microsomal cytochrome P-450 only those isozymes with the corresponding MAb-specific epitope, a result consistent with spectral measurements (Friedman et al., 1983). For the antigen exchange experiment carried out with each MAb, the activities of antigen-exchanged cytochrome P-450 (lines 3) greatly exceeded those of the glycine-eluted P-450s (lines 2), demonstrating that successful exchange has indeed occurred for all three MAb-purified cytochromes P-450. The yields of exchanged activities relative to that of total immunoadsorbed activities ranged from 3% to 12%. This variation in yield may arise from differential affinity of the different MAbs for the glycine-eluted P-450s and active cytochromes P-450, or from different dissociation rates of the immobilized cytochromes P-450 from the Sepharose–MAb matrices.

The primary objective of this study was to obtain significantly measurable levels of catalytic activities for the MAb-specific cytochromes P-450. Measurable activities toward two substrates were indeed obtained with this approach. In addition, yields may vary and improve with different MAbs or with other proteins. Further modifications of the basic antigen exchange procedure may also increase the yield of active cy-

tochrome P-450. The presence of glycine-eluted P-450 along with the exchanged, active cytochrome P-450 also does not detract from the utility of the antigen exchange method, since it is catalytically "silent" and does not directly contribute to the measured activity. It may, however, influence activity if it is capable of competing with active enzyme for binding to reductase.

We have thus demonstrated that MAb-specific cytochrome P-450 may be prepared in an active form by immunopurification. While immunopurification has previously proven useful for isolating cytochromes P-450 from different tissues and species, these have been inactive forms that, although quite suitable for primary structural studies, are inadequate for functional studies. With the antigen exchange technique, MAbs can also provide cytochromes P-450 that are suitable for catalytic characterization. The method is simple, should be readily applicable to a variety of tissues, and provides sufficient active cytochrome P-450 for a variety of catalytic studies, including evaluation of substrate and product positional selectivity of cytochrome P-450 isozymes.

Since the antigen exchange concept is general in nature, it may also be appliable to purification of other proteins for which functional activity of the enzyme or protein is required. The major prerequisite for successful antigen enchange is retention of structural integrity of the MAb-specific epitope. While the glycine-eluted P-450s obtained with three different MAbs indeed maintained some structural integrity after exposure to 0.1 M glycine (pH 3.0), other elution conditions may be more appropriate and will need to be evaluated for other proteins. In addition, the procedure conditions may vary with different MAbs, since the structural stability as well as efficiency in competing with native antigen may vary among different MAb-specific epitopes. With these considerations in mind, MAb-based immunopurification utilizing antigen exchange as an elution technique should be useful for the isolation of enzymatically or biologically active proteins that are otherwise obtained in an inactive state.

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