Turkey Gizzard Smooth Muscle Myosin Phosphatase-III Is a Novel Protein Phosphatase*

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Chromatography of turkey gizzard extract on Sephacryl S-300 has been shown to fractionate the various smooth muscle phosphatases. We have previously reported the purification and characterization of three of these enzymes, termed smooth muscle phosphatase (SMP)-I, -II, and -IV. Recently, we have purified SMP-III to near homogeneity. Although all of the smooth muscle phosphatases dephosphorylate the isolated myosin light chains, only SMP-III and -IV are active toward intact myosin and, therefore, are most likely to play a direct role in the muscle contraction-relaxation process. SMP-III has a higher molecular weight (390,000), as determined by gel filtration, than the other smooth muscle phosphatases and migrates as a single band with a molecular weight of 40,000 in a sodium dodecyl sulfate-polyacrylamide gel. SMP-III is immunologically distinct from SMP-I and -II. It dephosphorylates heavy meromyosin and the isolated myosin light chains at a rapid rate but has low activity toward phosphorylase α. The activity of SMP-III is not affected by Ca2+ but is activated by Mn2+. Mg2+ stimulates the activity toward heavy meromyosin but inhibits the myosin light chain phosphatase activity. Attempts to classify SMP-III according to the scheme proposed by Ingebritsen and Cohen (Ingebritsen T. S., and Cohen, P. (1983) Science 221, 331–338) revealed that it is resistant to the heat stable inhibitor-2, suggesting that it is a Type 2 protein phosphatase. However, SMP-III is inhibited by concentrations of okadaic acid which are characteristic of Type 1 protein phosphatases and it binds to heparin-Sepharose like other Type 1 phosphatases. But most interestingly, SMP-III does not dephosphorylate the α- or β-subunits of phosphorylase kinase, a property not reported for any Ser/Thr protein phosphatase.

Contractile activity is regulated primarily by the reversible phosphorylation of myosin (for review, see Refs. 1, 2). Studies on purified myosin from various smooth muscles revealed that unless the 20,000-Da light chains of myosin are phosphorylated on Ser-19, the actin-activated myosin MgATPase is low. A direct correlation exists between the extent of phosphorylation of myosin and the actin-activated MgATPase activity, the in vitro correlate of muscle contraction. In intact muscle fibers and permeabilized muscle preparations, myosin phosphorylation was observed to precede or occur simultaneously with tension formation following stimulation.

Myosin light chain kinase, the enzyme which catalyzes the phosphorylation of myosin has been purified from skeletal, cardiac, smooth muscles, and nonmuscle cells, and is well characterized (2, 3). On the other hand, the enzymes which catalyze the dephosphorylation of myosin are poorly understood. We have previously reported the purification to homogeneity and characterization of three protein phosphatases from turkey gizzards extract, termed smooth muscle phosphatase (SMP)-I, -II, and -IV, (4). A fourth phosphatase, termed SMP-III, has also been detected in the extract. All of these enzymes dephosphorylate the isolated 20,000-Da myosin light chains (MLC) but only SMP-III and -IV are active toward intact myosin. SMP-I is a Type 2A protein phosphatase according to the classification proposed by Ingebritsen and Cohen (5, 6). It is composed of three subunits (60, 55, and 38 kDa) in equimolar ratios, and the 38-kDa subunit was identified as the catalytic component of the enzyme (7). SMP-II, a Type 2C protein phosphatase, is a monomer (44 kDa) (5, 8). It is inactive in the absence of divalent cations and can be activated by Mg2+ and Mn2+. Ca2+ does not activate the enzyme but inhibits the Mg2+-activated activity of SMP-II (9). SMP-IV is a dimer (58 and 40 kDa) which does not fulfill the criteria for a Type 1 or 2 protein phosphatase because it dephosphorylates the α-subunit of phosphorylase kinase and is not inhibited by the heat stable inhibitor-2 (10). Binding studies of the smooth muscle phosphatases to actin and myosin filaments revealed that none of these phosphatases bind to actin and are not likely to be localized on the thin filaments (11). SMP-III and IV bound tightly to myosin, suggesting that they may be localized on the thick filaments in vivo.

Since SMP-III end -IV dephosphorylate intact myosin, they are most likely to be directly involved in the process of relaxation. In this paper, we report the purification of SMP-III to near homogeneity. SMP-III migrates as a single band (Mr = 40,000) on SDS-polyacrylamide gel but its molecular mass as determined by gel filtration is 390,000. Like SMP-IV, SMP-III cannot be classified as Type 1 or 2 protein phosphatase because it is not inhibited by the heat stable inhibitor-2, a property of Type 2 phosphatases, and is inhibited by concentrations of okadaic acid typical for Type 1 phosphatases. But more importantly, SMP-III does not dephosphorylate either the α- or β-subunit of phosphorylase kinase, a property which has not yet been reported for any Ser/Thr protein phosphatase.  

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*The abbreviations used are: SMP, smooth muscle phosphatase; SMP-I, protein phosphatase 2A1, composed of 40,000, 55,000, and 38,000 subunits; SMP-II, protein phosphatase 2C, a monomer of 43,000 polypeptide; SMP-IV, composed of 40,000 and 58,000 subunits; ML Ch, myosin light chains; HMM, heavy meromyosin; PMSF, phenylmethylsulfonyl fluoride.
Turkey Gizzard Smooth Muscle Phosphatase-III

MATERIALS AND METHODS

Resins for column chromatography, Sephacryl S-300, DEAE-Sephacel, CNBr-activated Sepharose, heparin-Sepharose, and Sephacryl G-200 were purchased from Pharmacia LKB Biotechnology Inc. while aminohexyl-agarose was obtained from Miles Laboratories. The re- 
combinant heat stable inhibitor-2 was a gift of Dr. Anna de Paoli-Roach (Indiana University) while the okadac acid was kindly provided by Dr. Akira Takai (Nagoya University). The rabbit skeletal muscle type I phosphatase, heat stable inhibitor-2, and phospho- 
phorylase a were gifts of Dr. Ramji L. Khandelwal (University of 
Kansaska). All other chemicals used were reagent grade.

Purification of SMP-III—Unless indicated, all procedures were carried out at 4 °C. Fresh turkey gizzards (360 g) were ground in a food processor and homogenized with (NH4)2S04. The homogenate was centrifuged at 20,000 g for 30 min, and the pellet was dissolved in and dialyzed against 20-500 mM KCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM PMSF, and then chromatographed on a heparin-Sepharose (1.5 × 5 cm) column. The column was equilibrated with the above buffer at 100 ml/h. The flow rate was adjusted to 2 ml/min. The fractions in low ionic strength solutions. Under these conditions myosin precipitates out, therefore, we preferentially use HMM to myosin in our study. The 20,000-Da MLC and HMM were phos- 
phorylated with turkey gizzard myosin light chain kinase as described in Ref. 7. Phosphorylase kinase and histone II A were phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (14).

Polyacrylamide Gel Electrophoresis—Gel electrophoresis was performed in the presence of SDS as described by Laemmli (15) was carried out in 12.5% microsac gel containing 0.1% SDS. Gel electrophoresis was performed in the absence of SDS was carried out in 3.5% polyacrylamide tube gels by a modified procedure described by Fairbanks et al. (16). The protein was eluted from the gel by cutting the gel into 2-mm slices and incubating each gel slice in 100 μl of 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 0.2 mg/ml of β-lactoglobulin, 1 mM dithiothreitol at 4 °C overnight. The eluates were assayed for phosphatase activity toward MLC and HMM.

Molecular Weight Determination—The molecular weight of SMP-III was determined by gel filtration on a Sephadex G-200 column (0.9 × 60 cm) equilibrated with 0.5 M NaCl, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol at 1 ml/h. The column was calibrated with the molecular weight protein standards (Pharmacia Fine Chemicals), thyroglobulin (689,000), feritin (200,000), catalase (232,000), aldolase (158,000), bovine serum albu- 
mun (67,000), ovalbumin (43,000), and chymotrypsinogen (25,000).

Tryptsin Digestion—Tryptsin-1-1-tryosyl-amido-2-phenethyl chloro- 
romethyl ketone (1 mg/ml) was added to SMP-III in 50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol to a final concentration of 5 μg/ml. The reaction mixture was incubated at 30 °C. Aliquots of the reaction mixture were taken at various time points and added to a solution of soy bean trypsin inhibitor to a final concentration of 50 μg/ml to 
terminate the reaction. After 15 min of incubation, the aliquots were 
assayed for phosphatase activities toward MLC and HMM. The control was SMP-III subjected to the same procedure except for the 
adition of water instead of trypsin.

Dephosphorylation of Phosphorylase Kinase—The time course of 
the activity of SMP-III toward phosphorylase kinase was determined by taking aliquots of the reaction mixture containing 32P-label- 
ed phosphorylase kinase (52 μg/ml) in 50 mM Tris-HCl, pH 7.4, 1 mM 
dithiothreitol, and with or without 32P-labeled MLC (1 mM) at various 
time points after the addition of SMP-III. The aliquots were mixed 
with equal volume of 0.1 M Tris-HCl, pH 6.8, 2% SDS, 2% glycerol, 
bromphenol blue, 1 mM mercaptoethanol and boiled immediately for 
5 min. The samples were applied to an SDS-polyacrylamide gel and the gel was autoradiographed.

RESULTS

Purification—As previously noted, most of the protein 
phosphatase activities toward MLC and HMM in turkey 
gizzard extract precipitated in the 30-60% (NH4)2SO4 satu- 
ration fraction (7). When this fraction was chromatographed on 
DEAE-Sephacel, the MLC and HMM phosphatases eluted as 
a single peak at 0.3-0.4 M KCl. Gel filtration of the active 
eluete on Sephacryl S-300 fractionated the protein phospha- 
tases into two peaks, one peak toward MLC and two peaks of 
MCL phosphatase activity, as previously reported (7). The 
second peak of HMM phosphatase activity coeluted with the 
first peak of MLC phosphatase activity. We have previously 
purified SMP-I and -IV from this peak and SMP-II from the 
second MLC phosphatase peak (7, 8, 10). The first peak of 
HMM phosphatase activity from the Sephacryl S-300 column 
was labeled as SMP-III and was further chromatographed on 
heparin-Sepharose. Fig. 1 shows that both phosphatase ac- 
tivities toward MLC and HMM coeluted at 0.4-0.5 M KCl. An 
8-fold purification was achieved in this step because SMP- 
III bound more tightly to the resin than most of the contam- 
inating proteins which eluted at lower ionic strength. The 
final step in the purification procedure was affinity chroma-
against 20 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and applied to a column of heparin-Sepharose. Although a 3-fold purification was achieved when the phosphatase was chromatographed in the absence of Mg\(^{2+}\), the enzyme preparation obtained after this step showed numerous bands on SDS-polyacrylamide gel. Rechromatography of the phosphatase preparation in the presence of Mg\(^{2+}\) resulted in further purification. After this step, the SMP-III preparation usually exhibits two bands (M\(_r\) = 40,000 and 46,000) on a SDS-polyacrylamide gel (data not shown). Table I shows that the purification procedure we have developed for SMP-III results in about 900-fold purification. The yield of enzyme from 360 g of turkey gizzards is about 30 \(\mu\)g which is comparable to that of SMP-IV but lower than that of SMP-I and -II. The SMP-III preparation stored in 20 mM KCl, 50 mM Tris-HCl, 1 mM dithiothreitol, 50% glycerol was stable at -20 °C for at least 6 months.

Physical Properties—To determine whether the two bands observed in the gel are associated with the phosphatase activity, the SMP-III preparation was subjected to gel electrophoresis under nondenaturing condition. The gel was then sliced and eluted. Fig. 2A shows the activity profile of the eluate of the gel slices. The phosphatase activities toward MLC and HMM comigrated as a broad peak, suggesting that both activities are inherent properties of SMP-III (Fig. 2A). Examination of these elutes on SDS-polyacrylamide gel revealed that only one band (M\(_r\) = 40,000) was observed in the active fractions which correlated with the phosphatase activity. Because the intensity of the protein bands in the Coomassie Blue-stained gel was weak, we silver stained the gel to improve the visualization of this band and to reveal other protein bands that might be associated with the activity of SMP-III. Fig. 2B shows the silver-stained gel of the eluates of gel slices 6–11. The other band observed in the SMP-III preparations (M\(_r\) = 46,000) is hardly visible in the active fractions. Two other bands (M\(_r\) = 57,000 and 70,000) are disclosed by silver staining, but their concentrations are very low compared to the 40,000-Da band except in the eluate of gel slice 10 (lane 5) and do not correlate with the phosphatase activity. Prior to silver staining, the Coomassie Blue-stained gel showed that the intensity of \(\beta\)-lactoglobulin (M\(_r\) = 18,000) in lane 5 was darker than the same band in the other lanes indicating that the volume of the eluate of gel slice 10 applied to this lane was more than the volumes of the eluates applied to the other lanes. This explains the increased intensity of the protein bands in this lane. We have repeated this determination five times with different SMP-III preparations and consistently observed the 40,000-Da band as the major component of the active fractions and its intensity in the Coomassie Blue-stained gel correlates with the phosphatase activity. Unfortunately, this difference in the intensity is lost upon silver staining of the gel. Our observations strongly suggest that SMP-III is composed mainly of the 40,000-Da protein. It has been shown that most Type 1 protein phosphatases share a common catalytic subunit (37,000) while Type 2A protein phosphatases have an identical 38,000-Da catalytic subunit (for review see Refs. 17, 18). Since the M of SMP-III is very close to these values, we routinely use SMP-I as molecular mass standard (Fig. 2B, lane 7) in addition to the Bio-Rad protein standards (lane 8) in SDS-polyacrylamide gels for more accurate comparison of the molecular weights. We always observe that SMP-III migrates slightly slower than the catalytic subunit of SMP-I suggesting that it is different from the catalytic subunits of Type 1 and 2A protein phosphatases reported.

The molecular weight of the holoenzyme enzyme was determined by chromatography of the purified SMP-III on a Sephadex G-200 column calibrated with molecular weight standard proteins, thyroglobulin, ferritin, catalase, aldolase, ovalbumin, bovine serum albumin, and chymotrypsinogen. This procedure revealed that SMP-III behaved like a globular protein with a molecular weight of 390,000 under nondenaturing conditions.

The possibility of a structural relationship between SMP-III and the other turkey gizzard phosphatases, SMP-I and -II, was verified by testing the cross-reactivity of SMP-III with the antibodies against SMP-II, and the 60- and 38-kDa sub-

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**Table I**

**Purification of SMP-III using HMM and MLC as substrates**

| Volume | Conc. | Myosin light chains | Heavy meromyosin | Specific activity | Total activity | -fold | Specific activity | Total activity | -fold |
|--------|-------|---------------------|------------------|-----------------|---------------|-------|-----------------|---------------|-------|
| ml     | mg/ml | nmol/min            | nmol/min/mg      | Purification    | nmol/min/mg   |       | nmol/min/mg     | Purification  |       |
| Extract| 1,200 | 7.8                 | 4680             | 0.5             | 1             | 5616  | 0.6             | 1             |
| 30-60% (NH\(_4\))\(_2\)SO\(_4\) | 93    | 71.5                | 5320             | 0.8             | 2             | 3324  | 0.5             | 1             |
| DEAE-Sepharose | 33    | 36.2                | 4420             | 3.7             | 7             | 2031  | 1.7             | 3             |
| Sephacryl S-300 | 107   | 0.3                | 244              | 7.6             | 15            | 321   | 10              | 17            |
| Heparin-Sepharose | 19    | 0.1                | 114              | 60              | 120           | 148   | 78              | 130           |
| Thiophosphorylated HMM-Sepharose | | | | | | | | |
| -Mg\(^{2+}\) | 24    | 0.02               | 88               | 186             | 372           | 101   | 211             | 351           |
| +Mg\(^{2+}\) | 1     | 0.03               | 14               | 466             | 932           | 15    | 500             | 833           |
units of SMP-I (12). None of these antibodies cross-reacted with SMP-III suggesting that it is distinct from SMP-I and -II (data not shown).

**Kinetic Properties**—SMP-III dephosphorylated MLC and HMM at a rapid rate. Using 1 μM of substrate in the assay, the specific activities of the enzyme toward MLC and HMM are 6.7 and 5 μmol/min/mg, respectively. The Lineweaver-Burk plots of the enzyme activities toward these substrates showed that the Km for MLC and HMM are 0.33 and 0.18 mM, respectively, while the V_max of their reactions are 21.6 and 20.7 μmol/min/mg, respectively. SMP-III exhibited very low activity toward rabbit skeletal muscle phosphorylase a and did not dephosphorylate phosphorylase kinase or histone IIA.

**Effectors**—The activities of SMP-III toward MLC and HMM were affected in different manners by the cations Ca^2+ and Mg^2+. Ca^2+ did not have any significant effect on the activities of SMP-III (data not shown) while Mn^2+ stimulated the enzyme activities (Fig. 3A). The activities were assayed for activity (A) toward MLC (●) and HMM (○). Panel B shows a silver-stained SDS-polyacrylamide gel of the eluates of gel slices 6–11 (lanes 1–6, respectively), SMP-I (lane 7), and Bio-Rad protein standards (lane 8).

Classification of SMP-III—To classify SMP-III according to the scheme proposed by Ingebritsen and Cohen (6), we studied the effect of the heat stable inhibitor-2 on its activity. The recombinant heat stable inhibitor-2 inhibited the activity of SMP-III to act on phosphorylase kinase was due to inactivation of the enzyme during the course of the experiment, we repeated the experiment in the presence of 32P-labeled MLC. The autoradiograph of a SDS-polyacrylamide gel showing the time course of the dephosphorylation of phosphorylase kinase in the presence of MLC revealed that MLC was almost Type I protein phosphatase from rabbit skeletal muscle but did not have any significant effect on the activities of SMP-III toward HMM and MLC suggesting that SMP-III is a Type 2 protein phosphatase. The same results were obtained when inhibitor-2 purified from rabbit skeletal muscle was used.

To eliminate the possibility that a protein present in the preparation might be interfering with the inhibition of SMP-III by inhibitor-2, we digested the enzyme with trypsin. Determination of the effect of inhibitor-2 on the activity of the proteolyzed preparation showed no change in the sensitivity of SMP-III to inhibitor-2 (data not shown). However, we observed that proteolysis stimulated the activity of the phosphatase toward HMM and MLC by 220 and 180%, respectively, immediately after the addition of trypsin (Fig. 5). The stimulation decreased gradually on prolonged digestion.

Fig. 6 shows that like most protein phosphatases, SMP-III is inhibited by okadaic acid. When this inhibition is compared to other protein phosphatas, we observed that the inhibition curve of both MLC and HMM phosphatase activities of SMP-III resembles that of Type 1 protein phosphatase rather than SMP-I, a Type 2A protein phosphatase suggesting that it is likely to be a Type 1 protein phosphatase.

Another criteria for the classification of the protein phosphatases is their ability to dephosphorylate the α- or β-subunits of phosphorylase kinase. We observed that SMP-III did not dephosphorylate either the α- or β-subunit of phosphorylase kinase. To determine whether this inability of SMP-III to act on phosphorylase kinase was due to inactivation of the enzyme during the course of the experiment, we repeated the experiment in the presence of 32P-labeled MLC. The autoradiograph of a SDS-polyacrylamide gel showing the time course of the dephosphorylation of phosphorylase kinase in the presence of MLC revealed that MLC was almost.
DISCUSSION

The gel filtration of the turkey gizzard extract on Sephadryl-300 is a crucial step in the purification procedure because it separated SMP-III from the other smooth muscle phosphatases, SMP-I, -II, and -IV. The observation that SMP-III eluted before the peak of SMP-I and -VI suggests that its molecular weight is higher than 160,000, the molecular weight of SMP-I, and/or that it is asymmetric. Indeed, chromatography of the purified SMP-III on a calibrated Sephadex G-200 column revealed that it eluted like a globular protein with a molecular weight of 390,000. Since only one protein (Mr = 40,000) was observed to be associated with SMP-III, it appears that the holoenzyme is a multimer of this protein. Other minor bands were observed in the silver-stained gel, but they did not correlate with the activity and were present in very low concentration compared to the 40,000-Da band. Whether other subunit(s) of SMP-IV was dissociated from the 40,000-Da protein during the experimental manipulation remains to be determined. It is interesting to note that one of the subunits of SMP-IV has a Mr of 40,000. Since both of these enzymes dephosphorylate intact myosin, it is possible that they share the same catalytic subunit. We have not yet established any structural relationship between these two enzymes but have shown that polyclonal antibodies against SMP-I and -II do not cross-react with either SMP-III or SMP-IV. Comparing the properties of SMP-III and -IV revealed other similarities and differences between these enzymes. Although both enzymes dephosphorylate the isolated MLC and HMM at a rapid rate, SMP-III appears to be more substrate specific. It has very low activity toward phosphorylase a and does not dephosphorylate phosphorylase kinase and histone IIA. The activities of both enzymes toward HMM are stimulated by Mg\(^{2+}\) while the MLC phosphatase activity is inhibited. This property was exploited in the final step of the purification procedure for SMP-III. Affinity chromatography on thiophosphorylated HMM-Sepharose in the presence of 5 mM Mg\(^{2+}\) improved the purification 3-fold over that in the absence of Mg\(^{2+}\). Ca\(^{2+}\) affected SMP-IV activity in the same manner as Mg\(^{2+}\) but it did not have any effect on SMP-III activity. Mn\(^{2+}\) stimulated both MLC and HMM phosphatase activities of SMP-III.

Ingvarsson and Cohen (6) reported that virtually all Ser/Thr protein phosphatases could be classified as Type 1 or 2. Type 1 protein phosphatases are those enzymes which are inhibited by the heat stable inhibitors-1 and -2, and dephosphorylate the \(\beta\)-subunit of phosphorylase kinase while Type 2 protein phosphatases are resistant to the inhibitors and act preferentially on the \(\alpha\)-subunit of phosphorylase kinase. SMP-III is not inhibited by heat stable inhibitor-2 suggesting that it is a Type 2 protein phosphatase. However, it exhibits other properties which are characteristic of Type 1 protein phosphatases. Okadaic acid is a potent phosphatase inhibitor isolated from marine sponges and has been used to differentiate Types 1 and 2A phosphatases because of the greater sensitivity of the latter to the inhibitor (19). We observe that the concentration-dependent inhibition curve for SMP-III is similar to that for Type 1 protein phosphatase from rabbit.
skeletal muscle. In contrast to SMP-I, the Type 1 protein phosphatase was found to bind tightly to myosin. To test this possibility, we treated SMP-I with trypsin to digest the target protein which is resistant to trypsin. Although the trypsin treatment increased about 50% while the myosin phosphatase activity of PP-I decreased about 50%. Therefore, both enzymes were treated with trypsin and the resulting 2-subunit enzyme or free catalytic subunit which could not be related to SMP-I but the difference in their activity toward myosin is not clear. It is possible that the enzyme preparation contains some 2-subunit enzyme or free catalytic subunit which could account for its activity toward myosin. Sobieszek and Barylko (28) also reported the purification of two MLC phosphatases from chicken gizzard. One enzyme has two subunits (Mr = 100,000 and 30,000) while the other enzyme shows a single band (Mr = 40,000) on SDS-polyacrylamide gel. The latter enzyme was purified from a peak of phosphatase activity from Sephacryl S-300 which was calculated to have a molecular weight of 360,000. It is likely that this enzyme is the same as SMP-I but the difference in their activity toward myosin is not clear. It is possible that this enzyme preparation contains some 2-subunit enzyme or free catalytic subunit which could account for its activity toward myosin. Sobieszek and Barylko (28) also reported the purification of two MLC phosphatases from chicken gizzard. One enzyme has two subunits (Mr = 100,000 and 30,000) while the other enzyme shows a single band (Mr = 40,000) on SDS-polyacrylamide gel. The latter enzyme was purified from a peak of phosphatase activity from Sephacryl S-300 which was calculated to have a molecular weight of 360,000. It is likely that this enzyme is the same as SMP-I but the difference in their activity toward myosin is not clear. 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