Redox Control of Calcineurin by Targeting the Binuclear Fe$^{2+}$-Zn$^{2+}$ Center at the Enzyme Active Site*

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The interaction of protein serine/threonine phosphatase calcineurin (CaN) with superoxide and hydrogen peroxide was investigated. Superoxide specifically inhibited phosphatase activity of CaN toward RII (DLD-VIPGRFDVVSVAAE) phosphopeptide in tissue and cell homogenates as well as the activity of the enzyme purified under reducing conditions. Hydrogen peroxide was an effective inhibitor of CaN at concentrations several orders of magnitude higher than superoxide. Inhibition by superoxide was calcium/calmodulin-dependent. Nitric oxide (NO) antagonized superoxide action on CaN. We provide kinetic and spectroscopic evidence that native, catalytically active CaN has a Fe$^{2+}$-Zn$^{2+}$ binuclear center in its active site that is oxidized to Fe$^{3+}$-Zn$^{2+}$ by superoxide and hydrogen peroxide. This oxidation is accompanied by a gain of manganous dependence of enzyme activity. CaN isolated by a conventional purification procedure was found in the oxidized, ferric enzyme form, and it became increasingly dependent on divalent cations. These results point to a complex redox regulation of CaN phosphatase activity by superoxide, which is modified by calcium, NO, and superoxide dismutase.

Calcineurin (CaN, also called protein phosphatase 2B) is a protein serine/threonine phosphatase regulated by calcium/calmodulin. The list of physiological CaN substrates is growing and its important role in such processes as T-cell activation, heart and skeletal muscle hypertrophy, synaptic plasticity, and apoptosis has been recognized (reviewed in Refs. 1 and 2). The significance of CaN for the activation of T-cells by depopshorylation of NFAT (nuclear factor of activated T cells) was revealed after the discovery of its inhibition by the immunosuppressive drugs cyclosporin A and FK506 (3). Recent interest has been focused on CaN regulatory mechanisms beyond calcium/calmodulin, and redox events have been suggested as modulators of phosphorylation/dephosphorylation pathways (4, 5). Among reactive oxygen species (ROS), superoxide (O$_2^-$) (6) and hydrogen peroxide (H$_2$O$_2$) (7–10) had been suggested as inhibitors of CaN activity, but the complex enzymology of CaN has led to conflicting conclusions about the mechanism of inhibition. With regard to the H$_2$O$_2$ effect, we have postulated a dithiol-disulfide transformation, since inhibition of the isolated enzyme was accompanied by the disappearance of two thiol groups, and reactivation was achieved by thioredoxin and di-thiols (10). Furthermore, CaN activity could be blocked by phenylarsine oxide, a selective agent for vicinal di thiols at low concentrations. However, the H$_2$O$_2$ concentrations required for inhibition were well above 100 M and, hence, out of a physiological range. Therefore, the previously suggested inhibition by superoxide (6) seemed to be a more relevant mechanism under physiological conditions, especially because we had obtained evidence that O$_2^-$ alone or in combination with the -NO radical can participate in a variety of signaling mechanisms (11).

In their pioneering report Klee and co-workers (6) propose that the metal catalytic center in CaN is targeted by superoxide. Similarly to related phosphatases PP1, PP2A, and purple acid phosphatases, CaN contains a binuclear metal center necessary for catalysis (12). Metal analysis indicated that iron and zinc are the components of this center (13, 14), but the iron oxidation state in native CaN is controversial (1, 2). Klee and co-workers (6) suggest the existence of superoxide-sensitive ferrous iron, but on the basis of spectroscopic and kinetic studies on isolated bovine CaN it was later suggested by Rusnak and co-workers (14, 15) that native CaN exists in a redox-insensitive Fe$^{3+}$-Zn$^{2+}$ form. A redox-sensitive Fe$^{3+}$-Fe$^{2+}$ form of CaN resembling the well characterized binuclear center in bovine spleen purple acid phosphatase was artificially obtained by metal exchange (14, 16). It was hypothesized that this form could also contribute to redox sensitivity of native CaN in tissue homogenates (9, 17). However, no evidence for the existence of such form in vivo has yet been obtained.

In the present work we attempted to clarify the mechanisms of CaN redox control with emphasis on the possible sensitivity of the enzyme toward O$_2^-$. Here we provide data supporting the presence of the Fe$^{2+}$-Zn$^{2+}$ center in native, catalytically active CaN, which can be efficiently oxidized and inactivated by nanomolar O$_2^-$ concentrations in a calcium- and calmodulin-dependent manner. NO acts as O$_2^-$ antagonist in protecting the enzyme against this inactivation. Thus, CaN joins the O$_2^-$/NO regulatory system and allows a link between phosphorylation/dephosphorylation events and redox control.

EXPERIMENTAL PROCEDURES

Materials—All chromatographic media for protein purification were from Amersham Biosciences, Inc. Xanthine oxidase (XO), catalase, and Cu,Zn-SOD were from Sigma. Stock solutions of potassium superoxide (KO$_2$) were made in MnSO$_4$ dried over 3 Å molecular sieves, and O$_2^-$ concentrations were determined by using e$_{505}$ = 2086 $M^{-1} cm^{-1}$. CaN isolation—Bovine brain CaN was isolated according to purification procedure of Sharma et al. (18) as described in Bogumil et al. (10). Porcine brain CaN purification involved the following important modifications: ammonium sulfate precipitation and Affi-Gel blue chromatography steps were omitted, and calmodulin affinity chromatography peak fractions were subjected to gel filtration on a Superdex 200 col-
um. 5 mM ascorbate was included in all purification buffers before the gel filtration step to avoid oxidative inactivation of CaN. CaN purity was checked by 14% SDS-PAGE, and protein concentrations were determined by densitometry of the CaN A-subunit bands using bovine CaN as a reference.

Preparation of Cell and Tissue Extracts—Jurkat or RAW 264.7 cells (0.5–1×10^6) were pelleted by centrifugation (12,000 × g, 5 min), washed with cold phosphate-buffered saline, and lysed in 75 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM TCEP, 0.2 mM EGTA, 50 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml soybean trypsin inhibitor, and 10 μg/ml leupeptin) by three cycles of freezing-thawing in liquid N2. The suspension was centrifuged at 10 min at 14,000 rpm and 4 °C, and the supernatant was collected. After measuring the protein with BCA reagent (Pierce), its concentration was adjusted to 0.5–1 mg/ml with the lysis buffer. Bovine cerebellum cortex was homogenized in the lysis buffer using Polytron tissue dispersion (Kinematica) and centrifuged for 10 min at 14,000 rpm and 4 °C. The supernatant was diluted after protein determination to 1 mg/ml with the lysis buffer.

RII Phosphorylation—RII peptide (DLDVPIGPRDFIRVSAAE, Bachem), corresponding to a portion of the regulatory RII subunit of protein kinase A, was serine-phosphorylated with [γ-^32P]ATP by using the catalytic subunit of protein kinase A. 20 μl of 7.8 mM RII stock solution was added to a kinase reaction mixture containing 50 mM triethanolamine-HCl, pH 7.5, 5 mM MgCl2, 0.5 mM ATP, 1 mM [γ-^32P]ATP, and 200 units of protein kinase A in a volume of 1 ml. The reaction was allowed to proceed for 2 h at 30 °C, and the phosphorylated peptide was purified on 1–ml Sep-Pak C18 columns (Waters) pre-equilibrated with 5 ml of 30% acetonitrile, 0.1% trifluoroacetic acid followed by 8 ml 0.1% trifluoroacetic acid. After loading of the reaction mixture, the column was washed exhaustively with 0.1% trifluoroacetic acid. The peptide was eluted with 30% acetonitrile, 0.1% trifluoroacetic acid, then lyophilized and re-suspended in a total volume of 0.5 ml of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mg/ml bovine serum albumin.

Phosphatase Assay—The assay was performed essentially according to Fruman et al. (19). The assay buffer consisted of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM TCEP, 1 mg/ml bovine serum albumin, 6 μM RII peptide, 1 μM okadaic acid, and 0.4 mM CaCl2. To 20 μl of assay buffer an equal volume of protein diluted in lysis buffer was added, and the reaction was conducted at 30 °C for 5–10 min. The reaction was stopped by adding 50 μl of 20% DOWEX 50-X8 (200–400 mesh, Bio-Rad) in 10% trifluoroacetic acid. The mix was centrifuged 6 min at 14,000 rpm, and 40 μl of supernatant were taken for Cherenkov counting. The amount of protein was adjusted so that the substrate consumption did not exceed 25%. Duplicate cpm values from the phosphatase assay were averaged, and the resulting value was adjusted by subtracting the counts in blanks lacking cell lysate. This was divided by the specific activity of the substrate to give picomoles of phosphate released. Finally, this was divided by the reaction time and the amount of protein to give values expressed as nmol phosphate/min/mg of protein. In the experiments with isolated CaN, 300 μl calmodulin was present in the assay, and TCEP was omitted from the buffers with no effect on CaN activity.

Limited Proteolysis of CaN—Purified porcine CaN (1 μM) was incubated in 50 mM Tris-HCl buffer containing 100 mM NaCl, 0.5 mM TCEP, and 5 mM ascorbate, pH 7.5, with 0.05 μg of trypsin for 5 min at 30 °C. The reaction was stopped by adding 0.2 μg of soybean trypsin inhibitor. The reaction was diluted into lysis buffer before incubation with XO. Parallel samples were subjected to SDS-PAGE to control the extent of proteolysis.

Quantitation of Superoxide Production—The amount of superoxide generated by the XO/hypoxanthine system was assayed by measuring the rate of SOD-inhibitable cytochrome c reduction. Phosphatase assay buffer (50 mM Tris-HCl, 50 mM NaCl, 0.1 mM EGTA, 0.2 mM CaCl2, 5 mM MgCl2, 0.5 mg/ml bovine serum albumin, and protease inhibitors, pH 7.5) containing 50 μM cytochrome c was used, and the increase of A550 was followed spectrophotometrically at 30 °C after the addition of various XO amounts in the presence of 100 μM hypoxanthine and 200 units/ml catalase. Under these conditions 1–5 milliunits/ml XO generated 0.2–1 μM O2·⁻/min.

Metal Analysis—Porcine CaN was buffer-exchanged on a Sephadex G-25 desalting column and fast protein liquid chromatography column into 50 mM Tris-HCl buffer, pH 7.4 (pre-treated with Chelex 100 chelating resin (Bio-Rad), to remove excess of metal ions). The metal content of the samples was determined by inductively coupled plasma mass spectrometry (Spurenanalytisches Laboratorium Dr. Baumann, Maxhütte, Germany).

EPR Spectroscopy—EPR spectra were recorded at 9.5 GHz (X-band) microwave frequency on a Bruker ESP300E spectrometer with a dual-mode resonator and peripheral equipment described elsewhere (20). Purified porcine CaN (with equivalent amounts of calmodulin added) was concentrated to 20 mg/ml in an Ultrafree-4 centrifugal device (Millipore). After recording the spectrum, the sample was thawed and incubated with 10 mM H2O2 in the presence of 1 mM CaCl2, 5 min at room temperature, and the spectrum was recorded again.

RESULTS

Superoxide Inhibition of CaN Activity in Cellular Extracts and in Isolated Form—Brain extracts contain a variety of phosphatases requiring the use of the [γ-^32P]phosphorylated RII peptide in conjunction with PPI/2A inhibitor okadaic acid for specific assessment of CaN activity. Under these conditions the effect of O2·⁻ on CaN activity was tested by adding the XO/hypoxanthine system. Catalase (200 units/ml) was also present to block the possible effects of H2O2. At a fixed concentration of 100 μM hypoxanthine the addition of increasing amounts of XO caused a progressive inhibition of CaN activity with half-maximal inhibition at about 1 milliunit/ml XO, which corresponds to 0.2 μM O2·⁻ generated per min under assay conditions (Fig. 1A). PPI/2A activity in the homogenate, measured as okadaic acid-sensitive [32P]phosphate release in the presence of EGTA, was not affected by XO/hypoxanthine (data not shown). The effect of O2·⁻ was not restricted to brain extracts, since CaN activity in lysates from Jurkat T-lymphocytes and RAW 264.7 macrophages was inhibited by XO/hypoxanthine to a similar extent (data not shown).

When bovine CaN was isolated as reported (10), its activity in initial experiments, contrary to the homogenate, was found to be insensitive to inhibition by XO/hypoxanthine (data not shown). During the calmodulin affinity chromatography step in the standard procedure of CaN isolation the protein mixture was in contact with high calcium concentrations. These conditions, according to Klee and co-workers (6) could result in loss of CaN activity due to O2·⁻-dependent oxidation, and this might be responsible for the insensitivity of the isolated bovine CaN to XO/hypoxanthine system. Because ascorbate was found to be protective against oxidative inactivation (6), we carried out the
purification procedure with 5 mM ascorbate addition to all buffers involved in CaN isolation. Porcine brain homogenates served as a starting material since bovine tissues were not available due to the bovine spongiform encephalopathy crisis in Germany. We noticed that the newly isolated porcine CaN behaved differently toward stimulation with Mn²⁺ ions (Table I). In the presence of calmodulin the bovine enzyme was activated about 10-fold by Mn²⁺ in the assay with 32P-labeled RII-peptide. In contrast, Mn²⁺ activated the porcine enzyme only about 3-fold. The gain of Mn²⁺ dependence was accompanied with a loss of XO sensitivity; thus, the bovine enzyme retained about ~25% of the activity after incubation with XO, and the porcine enzyme was inhibited to 5–10% that of controls (data not shown). Fig. 1B shows the concentration dependence of purified porcine CaN inactivation by the XO/hypoxanthine system. The IC₅₀ for XO at 0.05 milliunit/ml is 10-fold lower than IC₅₀ observed in brain homogenate, possibly reflecting O₂⁻ scavenging by other homogenate constituents.

Although the XO/hypoxanthine system is widely used for O₂⁻ generation, it also co-generates substantial amounts of H₂O₂. In addition, commercial XO preparations possess strong proteolytic activity. The presence of catalase, the dithiulfide reductant TCEP, and protease inhibitors in our assay system should minimize the effects of H₂O₂ and proteases. To prove that the inhibition by XO/hypoxanthine is caused by O₂⁻ the homogenate or isolated enzyme was incubated with XO and 100 mM hypoxanthine in the presence of 200 units/ml Cu,Zn-SOD (Fig. 2). In both cases, SOD protected CaN against inhibition by XO/hypoxanthine. Moreover, Cu,Zn-SOD also increased the basal rate of purified CaN activity. We observed that CaN was rapidly inactivated during the assay (data not shown), and SOD prevented this inactivation, thus resulting in apparent activation of the enzyme when net phosphate release during 5 min was counted. Protection exerted by SOD confirmed that O₂⁻ generated by XO/hypoxanthine was the inhibitory species. Additionally, omission of catalase from the assay mixture did not alter the extent of inhibition by XO/hypoxanthine (data not shown), indicating that H₂O₂ generation by this system was insufficient to cause CaN inhibition. Finally, bolus additions of as low as 100 mM KO₂ could significantly inhibit enzyme activity (data not shown).

Although H₂O₂ generation by the XO system was not responsible for CaN inhibition, we (10) and others (8, 9) show that CaN could be inhibited by H₂O₂. To investigate the effects of H₂O₂ under the same conditions as for XO/hypoxanthine treatment, we incubated porcine CaN with increasing H₂O₂ concentrations in the presence of Ca²⁺, calmodulin, and Cu,Zn-SOD to exclude O₂⁻-mediated enzyme inhibition. H₂O₂ caused a dose-dependent inhibition of activity of purified CaN (Fig. 3) with IC₅₀ ~ 50 μM (lower than previously found (10)), confirming that H₂O₂ could also be a CaN inhibitory species, although its effective concentrations are about 3 orders of magnitude higher than those of O₂⁻.

Calcium/Calmodulin Dependence of CaN Inhibition by Superoxide and Hydrogen Peroxide—In the work of Wang et al. (6) a link was made between calcium and O₂⁻ dependence of CaN inactivation. In our experimental setting we added XO/hypoxanthine directly into the RII assay mixture containing 0.1 mM free Ca²⁺. To investigate the calcium dependence of CaN inhibition by XO/hypoxanthine, we preincubated the porcine enzyme with 2 milliunits/ml XO and 100 μM hypoxanthine in the absence and presence of 1 mM calcium and/or 500 nM calmodulin before measuring the CaN activity with RII peptide. As shown in Fig. 4, calcium/calmodulin, but not calcium alone, significantly increased the inhibitory effect of XO/hypoxanthine. Even in the absence of XO, CaN activity decreased to 50% that of control after a 10-min incubation with calcium/calmodulin. A similar dependence was observed in brain homogenates (data not shown). Altogether, these results indicated that inhibition of CaN by O₂⁻ occurs in a calcium- and calmodulin-dependent manner.

Calcium/calmodulin-dependent CaN activation involves the displacement of the autoinhibitory domain from the enzyme active site, allowing substrate access to the catalytic center (21, 22). A similar activation could be achieved by limited proteolysis of CaN (23, 24), which removes the autoinhibitory and calmodulin-binding domains and renders the enzyme constitutively active in the absence of calmodulin. To examine if the removal of these domains also relieves the calcium/calmodulin dependence of O₂⁻ inhibition, we subjected porcine CaN to limited proteolysis by trypsin. A five-min incubation reduced the apparent molecular mass of the CaN A subunit from 60 to 42

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TABLE I

| Activity                        | Bovine CaN | Porcine CaN |
|--------------------------------|-----------|-------------|
| + CaCl₂                        | 0.3 ± 0.2 | 2.6 ± 0.4   |
| + CaCl₂/CaM                    | 7.5 ± 5.2 | 72.2 ± 8.4  |
| + CaCl₂/MnCl₂                  | 0.8 ± 5.6 | 72.4 ± 8.0  |
| + CaCl₂/CaM/MnCl₂              | 14 ± 86   | 213.6 ± 25.6|

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²The activity of the bovine enzyme varied from preparation to preparation, with some preparations showing about 100-fold activation by Mn²⁺ and only 25% inhibition by XO/hypoxanthine.
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Fig. 3. Concentration-dependent inhibition of CaN by hydrogen peroxide. Purified porcine CaN was preincubated for 5 min at 30 °C with increasing concentrations of H2O2 in the presence of 0.5 mM CaCl2, 500 nM calmodulin, and 500 units/ml Cu,Zn-SOD before phosphatase activity assay. Data represent the means ± S.D. of at least three independent experiments.

Fig. 4. Inhibition of CaN by XO/hypoxanthine is calcium/calmodulin-dependent. Purified porcine CaN was preincubated for 10 min in the presence or absence of 1 mM CaCl2, 500 nM calmodulin (CAM), and 2 milliunits/ml XO with 100 μM hypoxanthine. After the addition of 200 units/ml Cu,Zn-SOD, CaN activity was assayed for 5 min. Data represent the means ± S.D. of at least three independent experiments.

kDa and rendered CaN activity calmodulin-independent (data not shown). When the trypsinized enzyme was preincubated with 1 milliunit/ml XO and 100 μM hypoxanthine for 5 min a similar inhibition was observed in the absence as well as in the presence of 1 mM Ca2+ and 500 nM calmodulin, being 36.7 ± 4.2 and 46.1 ± 3.4% of control values, respectively. These data provide additional evidence that a calcium/calmodulin-dependent conformational change in CaN not only allows access of the substrate to the catalytic center but also makes it sensitive to O2 attack.

Interestingly, CaN inhibition by H2O2 was also calcium/calmodulin-dependent (data not shown), indicating that both ROS are likely to act only after release of spatial restrictions by the autoinhibitory domain.

**NO Antagonizes CaN Inhibition by Superoxide**—NO is considered an O2 antagonist since both species react with each other at an almost diffusion-controlled rate to form peroxynitrite, itself a potent oxidizing agent. We tested the effect of the NO donor DEA-NO, which has a half-life of 10 min at 30 °C and generates 1.5 mol of NO per 1 mol of decomposed DEAE-NO, on the activity of CaN in brain homogenates and that of isolated porcine CaN. Fig. 5 shows that 50 μM DEA-NO (a concentration that completely scavenges O2 generated by 5 milliunits/ml XO as determined by the cytochrome c test) alone had no effect on CaN activity of the homogenate but significantly increased the activity of the purified enzyme, reflecting enzyme protection against inactivation during incubation. When added together with XO/hypoxanthine, DEA-NO protected CaN against inhibition. This indicates that NO serves as an O2 antioxidant under conditions where O2-mediated inhibition of CaN would take place. In contrast, DEA-NO could not protect CaN against inhibition by 100 μM H2O2 (data not shown), confirming that NO acts solely as a selective O2 scavenger. Additionally, peroxynitrite formed by the reaction of O2 and NO obviously does not exert any inhibitory effect, at least at the XO and DEA-NO concentrations applied.

**Superoxide Targets the Binuclear Metal Center of CaN**—Superoxide has been shown to react with various functional groups in proteins, including iron-sulfur clusters and protein thioles either in free state or metal-complexed, as in zinc fingers (25–27). In our previous work we could attribute inactivation of bovine CaN by phenylarsine oxide and H2O2 to the oxidation of protein cysteines (10). However, no thiol oxidation by O2 seems to be responsible for CaN inhibition since our assay system contained 0.5 mM TCEP, an efficient disulfide-reducing agent. In addition, 1 or 5 mM dithiothreitol or 10 mM reduced glutathione did not influence XO/hypoxanthine inhibition of CaN activity (data not shown). Thus, O2 appears to inactivate CaN by some other mechanism.

An obvious possibility could be an interaction of O2 with the iron-containing binuclear metal center of CaN, since O2 could, depending on the ligand field, either reduce ferric iron or oxidize ferrous iron with subsequent loss of catalytic activity. As mentioned before, the reduced state of iron in CaN is under dispute (6, 9, 15, 17). Metal content analysis of porcine CaN by inductively coupled plasma mass spectrometry showed the presence of equivalent amounts of iron and zinc in the protein (data not shown). To investigate the involvement of the metal center in the redox sensitivity of CaN, we treated the enzyme with 1 mM univalent oxidant K3Fe(CN)6 or with the same concentrations of K3Fe(CN)6 and KCl (Fig. 6A). K3Fe(CN)6...
inhibited CaN activity to 17% that of controls, whereas K2Fe(CN)6 and KCN had only slight inhibitory effects, suggesting that the inhibitory effect of K2Fe(CN)6 is due to enzyme oxidation. We also tested the effect of 5 mM ascorbate with or without XO/hypoxanthine on CaN activity (Fig. 6B). Ascorbate increased basal phosphate ester hydrolysis by CaN and protected the enzyme against inhibition by XO/hypoxanthine. Thus, CaN is inhibited by one-electron oxidizing agents and protected against O2•− inhibition by the reductant ascorbate. This indicates that CaN is likely to require Fe3+ for optimal activity.

The strong reductant dithionite was previously shown to inhibit CaN activity, interpreted as a requirement for Fe3+ in the catalytically active enzyme (14, 15). In our hands, however, direct addition of up to 10 mM dithionite to the assay did not inhibit the enzyme; rather, it caused apparent activation to 154 ± 6% of control, again indicating the presence of reduced iron in the active CaN.

Next, we investigated whether the O2•−-inactivated enzyme could be reactivated. In previous work of Wang et al. (6) it was found that the treatment of the calcium-inactivated enzyme with an Fe3+/ascorbate/dithiothreitol mix restored the enzyme activity in brain homogenates. We tested several combinations of metal ions for the ability to reactivate CaN after XO/hypoxanthine treatment (Fig. 7A). Ascorbate alone restored the activity of the enzyme to ~70% of the control value, and addition of 0.5 mM (NH4)2Fe(SO4)2 with 5 mM ascorbate fully reactivated the enzyme. The inhibition of CaN by 100 μM H2O2 could also be reversed by Fe2+/ascorbate to 90% of that of the control activity, suggesting that lower concentrations of H2O2 (<100 μM) also target Fe2+ in the binuclear cluster. At higher H2O2 concentrations (>100 μM) only partial reversibility could be achieved (data not shown), suggesting that other protein functional groups, such as previously identified thiols (10), are targeted by higher concentrations of H2O2.

Manganese II ions were shown earlier not only to increase CaN activity but also to restore it after calcium/calmodulin-mediated inactivation, although the mechanisms involved were poorly understood (13). We attempted to determine if Mn2+ could restore CaN activity after inhibition by O2•−. Fig. 7B shows that after incubation with 1 milliunit/ml XO in the presence of 100 μM hypoxanthine, porcine CaN was inhibited to 17% of control, and this inhibition could be reversed by 1 mM Mn2+ with activities being 65% of control values (in the presence of Mn2+). Remarkably, the addition of the Fe3+ chelator desferrioxamine further increased the ability of Mn2+ to restore enzyme activity up to 89% of control. Desferrioxamine alone had no effect on the enzyme activity or on inhibition by O2•−. The effect of desferrioxamine likely may be facilitation of iron removal from the cluster after its oxidation to Fe3+, and it indicated that Mn2+ could probably substitute for iron in the enzyme binuclear cluster. The loss of iron upon enzyme oxidation was confirmed by metal analysis (inductively coupled plasma mass spectrometry), which showed that approximately half of the enzyme iron is lost after O2•− treatment in the presence of calcium/calmodulin (data not shown).

**EPR Spectroscopy of Porcine CaN**—The low temperature EPR spectrum of native porcine CaN (0.25 mM) is shown in Fig. 8A. Its main feature consists of a weak signal around g = 4.3. Upon incubation of the CaN sample with 10 mM H2O2 in the presence of calcium/calmodulin this signal was significantly increased, and appeared as a doublet (Fig. 8B). Additional features centered at g = 6.5, 5.6 and 5.0 were present, of which the first two were previously assigned to a Fe3+ species with near axial symmetry (10, 15). The absence of these signals in
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fig. 8. EPR spectroscopy of porcine CaN. EPR spectra of purified porcine CaN before (A) and after (B) treatment with 10 mM H$_2$O$_2$ in the presence of 0.25 mM calmodulin and 1 mM CaCl$_2$. The EPR samples contained 20 mg/ml CaN in 50 mM Tris-HCl, 100 mM NaCl, 0.5 mM EGTA, and 0.5 mM TCEP, pH 7.5. Spectrometer conditions: temperature, 8K; microwave frequency, 9.66 GHz; modulation amplitude, 10 G at 100 kHz; microwave power, 2 mW.

the native sample provides further evidence for the presence of a Fe$^{2+}$-Zn$^{2+}$ center in native, catalytically active CaN.

**DISCUSSION**

The results of this study provide kinetic and spectroscopic evidence that native CaN has a catalytically active Fe$^{2+}$-Zn$^{2+}$ binuclear cluster. This cluster is particularly sensitive to O$_2^*$ and its inhibition is calcium- and calmodulin-dependent. Furthermore, NO antagonizes the O$_2^*$ inhibitory action, adding another player to the CaN regulatory network.

A role of ROS as modulators of CaN activity has been proposed lately (17). O$_2^*$ and H$_2$O$_2$ are considered major ROS generated in living cells; however, the potential of O$_2^*$ as a physiological oxidant until recently remained underestimated due to its instability and, therefore, low endogenous levels. Superoxide must also be regarded as a ROS with low reactivity. However, some new findings indicated that O$_2^*$ could be a much more effective and selective oxidant as H$_2$O$_2$ for several key components of intracellular signaling (11, 26, 27). Comparison of O$_2^*$ and H$_2$O$_2$ as CaN inactivators in our work showed that the former is effective at concentrations at least 3 orders of magnitude lower than the latter. Superoxide sensitivity of CaN is high enough to be of physiological importance and is in agreement with an earlier report of Klee and co-workers (6) on the inhibitory action of ROS.

Among possible targets of ROS at the protein, the most likely candidates are its binuclear metal center and its thiols. Although in our previous work we attributed CaN inhibition by H$_2$O$_2$ to oxidation of protein cysteines (10) we have no indication that CaN thiols play a major role in enzyme sensitivity to O$_2^*$. The link between redox regulation of CaN activity and the status of the enzyme binuclear metal center had recently become a matter of intensive discussion (2, 17, 33). Among the protein serine/threonine phosphatases, the nature of the metals in the binuclear center was clearly defined only for CaN, as iron and zinc (13, 14). The question of which oxidation state of iron is present in the native, active form of CaN remained unsolved. This study provides several lines of evidence in support of a binuclear Fe$^{2+}$-Zn$^{2+}$ center in native CaN. First,
native CaN was inhibited by Fe$^{3+}$, whereas Fe$^{2+}$ had no effect. Second, ascorbate could partly reactivate the O$_2^-$-inhibited enzyme, and Fe$^{2+}$/ascorbate could fully reverse it. The loss of iron after enzyme oxidation could explain the discrepancy between ascorbate and Fe$^{2+}$/ascorbate effects. Third, dithionite was not inhibitory when added directly into the assay in concentrations up to 10 mM. It also did not prevent the reactivation by Fe$^{2+}$/ascorbate (data not shown). Inhibition of CaN by dithionite provided the basis for assuming an Fe$^{3+}$-Zn$^{2+}$ cluster as the active enzyme form (14, 15). We found that under certain conditions, e.g., after aerobic preincubation, dithionite could indeed inhibit CaN activity. This could be partly attributed to radical generation during dithionite oxidation. Furthermore, we found that sulfite in concentrations of 0.1–1 mM also inhibited CaN. Because sulfite is unavoidable generated from dithionite, this factor should be taken into account when considering dithionite effects. The other difference between our data and previously published data is the use of III versus p-nitrophenyl phosphate as phosphatase substrates.

Last, EPR spectroscopic data do not support the presence of ferric iron in native CaN. A weak signal around $g = 4.3$, corresponding to highly rhombic high spin Fe$^{3+}$, was present in the native sample. This feature has been described in previous reports and was attributed either to adventitious iron (14, 15) or to iron in a binuclear Fe$^{3+}$-Zn$^{2+}$ center (10). The EPR spectrum of the oxidized sample was in general similar to the previously published EPR spectrum of bovine CaN (10). The two new features centered at $g = 6.5$ and 5.6 after H$_2$O$_2$ oxidation have been previously assigned to a Fe$^{3+}$-species with a near axial symmetry (10, 15) and most likely originate as primary oxidation products in the binuclear center. The previously described feature at $g = 8.1–8.5$ only weakly appears in the present spectrum, probably since a higher recording temperature was used, and this signal was reported to rapidly decrease with rising temperature (15).

We therefore agree with the hypothesis first proposed by Qin et al. (34) that the active CaN is likely to have a Fe$^{3+}$-Zn$^{2+}$ center. Drawing analogy to the Fe$^{3+}$-Zn$^{2+}$ state of purplish acid phosphatases appears to be not valid in this case since their iron ligand sphere contains a tyrosine, which tends to stabilize Fe$^{3+}$; whereas CaN contains a histidine, which favors the divalent iron state.

With regard to the mechanism of the O$_2^-$ action one also should consider the Lewis acids properties of the neighboring zinc ion, which can stabilize a peroxy- intermediate arising from the Fe$^{2+}$-O$_2^-$ interaction. This additional bridging would allow the ferrous species to acquire an oxidation potential high enough not to react with molecular oxygen. We therefore propose that the resulting species from O$_2^-$ inactivation would be a peroxy-bridged Fe$^{3+}$-O$_2^-$-Zn$^{2+}$ complex, which we now try to identify by magnetic circular dichroism spectrometry.

Our results also provide a link between manganese dependence of CaN activity and O$_2^-$-mediated inactivation of the enzyme. We suggest that the enzyme in its native state is manganese-independent. During purification, particularly affinity chromatography on calmodulin-Sepharose, the enzyme undergoes oxidative inactivation, which is accompanied by partial iron loss. The addition of ascorbate in enzyme purification buffers allowed the isolation of the reduced enzyme form. Because dithiothreitol was not protective against enzyme inactivation by O$_2^-$, the use of this agent as reductant in established purification procedures (18) is probably not effective. Some conventional bovine CaN preparations could be activated up to 100-fold by manganese and were not sensitive to superoxide. We observed that after inactivation by O$_2^-$, Mn$^{2+}$ could reactivate the enzyme, and this reactivation increased in the presence of the Fe$^{3+}$ chelator desferrioxamine, thus suggesting that Mn$^{2+}$ could substitute for iron in the enzyme active center. The enzyme activity in the presence of Mn$^{2+}$ was still higher than that of the control even for the intact enzyme or for the iron/ascorbate-reactivated enzyme, probably due to initial inactivation of the enzyme or to the presence of iron-free enzyme. In addition, some zinc loss could also take place. The reasons for such losses are unknown, although a similar O$_2^-$-dependent zinc release was documented for endothelin-converting enzyme (35). The gain of CaN Mn$^{2+}$ dependence during purification was noticed as early as in 1982 (36), and the interaction of Mn$^{2+}$ with CaN was investigated in detail (13, 37), but the present study is to our knowledge the first to connect Mn$^{2+}$ dependence to a previous oxidative inactivation of the enzyme.

In summary, the isolation of the redox-sensitive Fe$^{3+}$-Zn$^{2+}$ form of CaN confirms the initial hypothesis of Huang and co-workers (34) that this form represents the native enzyme. Our data provide evidence that native CaN could be the subject of complex redox regulation involving superoxide, calcium, NO, and SOD. Further studies are needed to shed more light onto the mechanism of phosphoester hydrolysis by CaN, which should be readapted to ferrous versus ferric iron in the enzyme binuclear metal center.

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Redox Control of Calcineurin by Targeting the Binuclear Fe$^{2+}$-Zn$^{2+}$ Center at the Enzyme Active Site
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