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^{31}P NMR Study of the Interaction of Inorganic Phosphate with Bovine Copper-Zinc Superoxide Dismutase

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Paramagnetic effects on ^{31}P phosphate resonances caused by Cu(II) ions in native and phenylglyoxal-modified bovine Cu,Zn-superoxide dismutase have been used to monitor the interaction of phosphate with these proteins. T_2 values are found to be 70 times smaller than T_1, indicating that some mechanisms, as yet undefined, contribute to the line width. Using T_1 measurements, we have determined that the affinity constants for phosphate binding to the native protein are 20 ± 4 and 34 ± 3 M^{-1} at pH 8.0 and 7.0, respectively, and that the Cu(II)-phosphate distance is 5.3 Å. At pH 6.3, two binding sites are observed, one at a distance >7 Å with an affinity constant >100 M^{-1} and another at approximately 5 Å with an affinity constant of 10 M^{-1}. Modification of the protein with phenylglyoxal causes the affinity of phosphate for the same sites to decrease by a factor of 3 at pH 6.3. These results indicate that phosphate does not bind directly to Cu(II) but to a site close by. We conclude that the site of phosphate binding is Arg-141, which is known from X-ray structural evidence to be located approximately 5 Å from the copper center.

Introduction

Bovine erythrocyte Cu,Zn-superoxide dismutase (Cu,Zn-SOD) [EC1.15.1.1] is a metalloenzyme of mol wt 31 200 that is comprised of two identical subunits, each of which contains one copper and one zinc ion in close proximity. It is a particularly well characterized metalloenzyme system[1] in that its amino acid sequence and X-ray crystal structure are known, the latter to 2-Å resolution.[2,3] This enzyme is found in the cytosol of almost all eukaryotic cells and in some bacteria and has been proposed to function in the cell as a protective agent against the effects of superoxide.[4]

X-ray crystal structural studies of bovine Cu,Zn-SOD indicate that the positively charged side chains of Arg-141, Lys-120, and Lys-134 are located in the vicinity of the active site, 5, 12, and 13 Å, respectively, away from the copper ion.[2,3] It is believed that these residues are present in predominantly hydrophobic portions of the channel that provides access to the active site and that they provide electrostatic guidance to the substrate, superoxide, and other anions.[5,6]

The site of reactivity for superoxide with the oxidized form of the enzyme is the copper(II) ion, which is also the binding site for several anions, as indicated by pronounced spectral shifts observed upon anion binding.[1] Inhibitory effects of these anions have been attributed to this type of binding.[1] Phosphate has also been shown to interact with bovine Cu,Zn-SOD,[7-10] but no visible or ESR spectral changes were observed in solutions of Cu,Zn-SOD even at high concentrations of phosphate.[11] In an earlier paper,[11] it was reported that the SOD activity of native bovine Cu,Zn-SOD, as well as its affinity for cyanide and azide, measured at constant ionic strength, decreased significantly in the presence of increasing concentrations of phosphate. When these experiments were repeated with Cu,Zn-SOD that had been chemically modified at Arg-141 with phenylglyoxal, by contrast, little dependence of either SOD activity or anion binding on phosphate concentration was observed. These results were interpreted as indicating that the inhibitory effect of phosphate on bovine Cu,Zn-SOD was due primarily to binding of phosphate to the side chain of Arg-141 with consequent neutralization of its positive charge. A preliminary study using ^{31}P NMR was also reported in which line broadening of phosphate resonances of inorganic phosphate and adenine nucleotides caused by the paramagnetic Cu(II) center was described.[12-14]

In this paper, we present a quantitative analysis of the binding of inorganic phosphate to bovine Cu,Zn-SOD. Paramagnetic effects on ^{31}P phosphate resonances caused by copper(II) ions present at the active site of native and chemically modified proteins have been used to monitor the interaction of phosphate with these proteins, and ^{31}P T_1 measurements have allowed us to discriminate between direct metal-to-phosphate binding and binding to other locations within 10 Å.[15]

Experimental Section

Bovine liver Cu,Zn-SOD was purchased as a lyophilized powder from Diagnostic Data, Inc. (Mountain View, CA). Phenylglyoxal, sodium azide, HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and sodium dithionite were purchased from Sigma, potassium phosphate dibasic trihydrate was purchased from Mallinckrodt, Inc., and deuterium oxide (99.8%) was from Cambridge Isotope Laboratories. All were used as received.

Bovine Cu,Zn-SOD was chemically modified at Arg-141 with phenylglyoxal by the method of Malinowski and Fridovich.[16] Protein concentrations were determined by using the absorbances at 260 nm (ϵ = 10 300 M^{-1} s^{-1}) and 680 nm (ϵ = 300 M^{-1} s^{-1}).[1] The two values always agreed within 5%. Native and Arg-modified SOD were reduced by addition of sufficient sodium dithionite to bleach the color of the protein.

^{31}P NMR measurements were made either at 32.4 MHz on a Varian FT80 or at 36.4 MHz on a Bruker CXP90 spectrometer at the ambient probe temperature (~30 °C). Some of the measurements were repeated at 121.3 MHz on a Bruker CXP300 NMR spectrometer. Longitudinal

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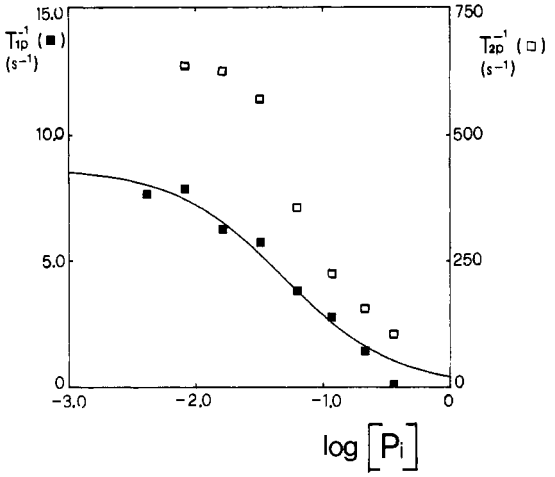

Figure 1. Dependence of T_{1p}^{-1} and T_{2p}^{-1} values of the ^{31}P signal of phosphate solutions in the presence of Cu,Zn-SOD as a function of phosphate concentration at pH 8.0. The measurements were performed at 36.4 MHz. The enzyme concentration was 0.23 mM, and the phosphate concentrations varied from (4–5) × 10^{-3} to 0.4–0.7 M.

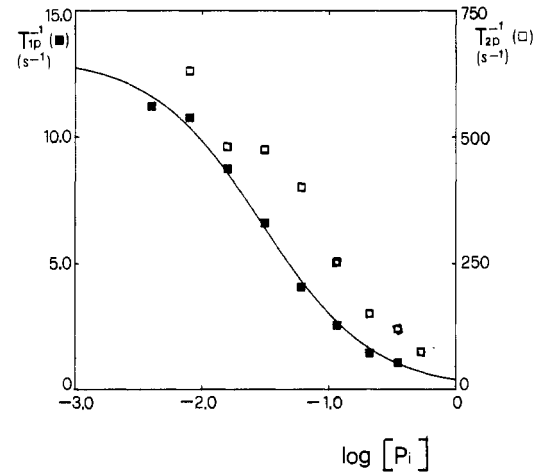

Figure 2. Dependence of T_{1p}^{-1} and T_{2p}^{-1} values of the ^{31}P signal of phosphate solutions in the presence of Cu,Zn-SOD as a function of phosphate concentration at pH 7.0. The experimental conditions were the same as in Figure 1.

relaxation times, T_1, were measured with the inversion recovery method using an appropriate nonlinear least-squares fitting program. Transverse relaxation times, T_2, were obtained from the line width at half-height, decreased by the line-broadening contribution resulting from exponential filtering, through the relation $T_2^{-1} = \pi\Delta\nu$. (These values were confirmed by a spin-echo experiment.) pH values quoted are not corrected for the deuterium isotope effect. ^{31}P NMR spectra were measured with 4000-Hz spectral width by using 8K data points.

Results

The binding of phosphate anion to bovine Cu,Zn-SOD was followed by the changes in the nuclear relaxation rates of the ^{31}P nucleus of phosphate as measured by 32.4- or 36.4-MHz ^{31}P NMR. Experiments were performed at pH 8.0, 7.0, and 6.3 in the presence of the native protein and pH 6.3 in the presence of the phenylglyoxal-modified protein. ^{31}P T_1 and T_2 values were measured at SOD concentrations of 0.16–0.23 mM and phosphate concentrations ranging from (4–5) × 10^{-3} to 0.4–0.7 M. In all instances, the T_1^{-1} and T_2^{-1} values were drastically increased with respect to analogous solutions containing the reduced proteins or no proteins at all, the latter two being almost indistinguishable. We conclude, therefore, that the increase in nuclear relaxation rates is entirely due to the interaction of phosphate with the paramagnetic copper(II) center in the active site. The paramagnetic contributions toward the relaxation rates, T_{1p}^{-1} and T_{2p}^{-1}, obtained by subtracting from the experimental data on the oxidized protein solutions those of the corresponding reduced protein solutions, are shown in Figures 1–4. The first striking feature is the much larger T_{2p}^{-1} with respect to T_{1p}^{-1} values at every phosphate concentration.[14] This result indicates that T_{1p}^{-1} is not determined by τ_M^{-1}, the exchange rate between bound and free phosphate. Otherwise, T_{1p}^{-1} and T_{2p}^{-1} would be equal and equal to $f_M\tau_M^{-1}$, where f_M is the molar fraction of bound phosphate. Therefore the upper limit of τ_M^{-1} is $(f_M T_{1p})^{-1}$, which is equal to 10^2 s^{-1}.[15] Since in all cases the relaxation parameters are concentration dependent, the T_{1p}^{-1} data can also be used to estimate the affinity of phosphate for the protein. The T_1 data have been used for this purpose because we believe that we understand their significance, but similar values are obtained by analyzing the T_2 data.

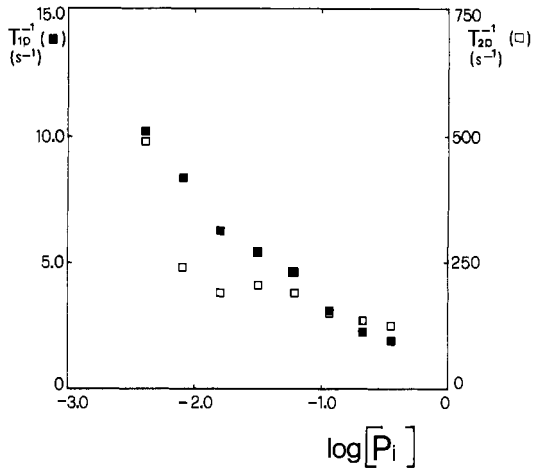

Figure 3. Dependence of T_{1p}^{-1} and T_{2p}^{-1} values of the ^{31}P signal of phosphate solutions in the presence of Cu,Zn-SOD as a function of phosphate concentration at pH 6.3. The measurements were performed at 32.4 MHz. The enzyme concentration was 0.16 mM and the phosphate concentrations varied from (4–5) × 10^{-3} to 0.4–0.7 M.

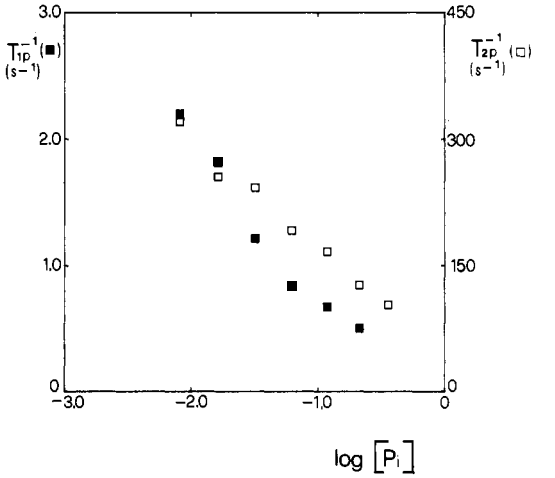

Figure 4. Dependence of T_{1p}^{-1} and T_{2p}^{-1} values of the ^{31}P signal of phosphate solutions in the presence of phenylglyoxal-modified Cu,Zn-SOD as a function of phosphate concentration at pH 6.3. The experimental conditions were the same as in Figure 3.

The data at pH 8 and 7 for the native protein show a simple sigmoidal behavior (Figures 1 and 2), indicating the presence of a single binding site for phosphate within the sphere of influence of the paramagnetic copper ion. The concentration dependence is satisfactorily fit by the following equation for the simple equilibrium enzyme + phosphate ⇄ enzyme–phosphate complex:

$$T_{1p}^{-1} = C_E[T_{1M}^{-1}K/(1 + KC_I)] \quad (1)$$

where C_E and C_I are the enzyme and phosphate concentrations, respectively, K is the affinity constant of phosphate for its binding site, and T_{1M}^{-1} is the relaxation rate of bound phosphate. The best fit values are $K = 20 \pm 4$ and 34 ± 3 M^{-1} and $T_{1M}^{-1} = 900 \pm 140$ and 836 ± 50 s^{-1} at pH 8.0 and 7.0, respectively. Although

the affinity changes somewhat on passing from pH 8 to 7, T_{1p}^{-1} changes accordingly so that the T_{1M}^{-1} values turn out to be the same within experimental error.

The analysis of the relaxation data in terms of the possible contributing mechanisms may provide information about the phosphate binding site. Both $(f_M T_{1p})^{-1}$ and $(f_M T_{2p})^{-1}$ contain a dipolar contribution, which is given by the coupling between the unpaired electron located on the metal and the ^{31}P nucleus. Such a mechanism is well understood theoretically and can be used to infer structural information. The ratio between the dipolar contributions to $(f_M T_{2p})^{-1}$ and $(f_M T_{1p})^{-1}$ can be calculated if the correlation time τ_c for the coupling is known. In this case, the correlation time has been independently measured on the free enzyme through water ^1H nuclear magnetic resonance dispersion measurements and found to be the electronic relaxation time of copper(II) in the compound (1.8×10^{-9} s at room temperature).[17] If it is assumed that this time is the same in the phosphate adduct and in the free enzyme, the above ratio is 1.27. Since every further mechanism operating on T_1^{-1} or T_2^{-1} or both leads to additional contributions to the rates, it follows that T_2^{-1}, being much larger than T_1^{-1}, cannot be determined solely by the dipolar relaxation mechanism. Therefore we use only the T_1^{-1} data to deduce structural information.

The T_{1M}^{-1} values obtained from eq 1 can be related to the metal–phosphorus distance, r, through the Solomon equation (eq 2). In this equation, τ_c is 1.8×10^{-9} s (see above), μ_0 is the

$$T_{1M}^{-1} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left(\frac{7\tau_c}{1+\omega_S^2\tau_c^2} + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right) \quad (2)$$

permeability of vacuum, r is the distance between the ^{31}P atom of phosphate and the copper(II) ion, γ_N is the nuclear magnetogyric ratio, g_e is the electron g factor, μ_B is the Bohr magneton, S is the spin quantum number, and ω_I and ω_S are the nuclear and electronic Larmor frequencies. It has already been shown that this equation is valid for copper(II) systems at the magnetic fields of the present experiments (1.88–2.11 T).[17] The estimated distance is 5.3 Å. Such a value indicates that phosphate is not directly bound to the copper ion and confirms a posteriori the assumptions of purely metal-centered dipolar interaction (i.e. no spin delocalization contributions through chemical bonds) and of the constancy of the electronic relaxation time, since the copper chromophore is not directly perturbed, as already suggested from electronic and EPR spectroscopies.[11] Furthermore, the ^1H NMR spectra of Cu,Co-SOD (a derivative of Cu,Zn-SOD in which Zn(II) has been replaced by Co(II)) measured in the presence of excess phosphate are absolutely identical with the spectra in the absence of phosphate. Such spectra display the signals of all of the protons of the histidyl imidazole ligands coordinated to both cobalt and copper and thus are highly sensitive to changes in coordination of the metal centers.[18] This experiment shows that phosphate does not alter the coordination sites. It should also be noted that a similar study of the interaction of phosphate with copper(II)-substituted carbonic anhydrase under conditions comparable to those described here for Cu_2Zn_2SOD has been reported.[19] In that case, phosphate is believed to bind directly to copper and, accordingly, $(f_M T_{1p})^{-1}$ values were observed that were 20 times larger than those reported here for Cu_2Zn_2SOD.

The T_{1p}^{-1} data at pH 6.3 (Figure 3) show a biphasic behavior that cannot be fit by eq 1. The pattern is suggestive of the presence of two anion binding sites with different affinities. Assuming that the two sites are not interacting, i.e. that binding of phosphate to one site does not significantly alter the affinity of the other site for phosphate nor its interaction with the paramagnetic center, the data indicate a site at a distance greater than 7 Å from the metal with an affinity constant greater than 100 M^{-1} and another at ~5 Å with an affinity of 10 M^{-1}. The latter site is likely to be the same as that characterized at higher pH values.

Analogous behavior is shown by phosphate interacting with the phenylglyoxal-modified protein at the same pH (Figure 4). The curve is lower, however, and shifted to higher phosphate concentration, indicating that the ^{31}P NMR data are probably monitoring the same sites as in the native enzyme and that the affinity for both of them is reduced by about a factor of 3. A more quantitative analysis of the pH 6.3 data would be difficult to attempt and is probably not worthwhile in view of the fact that the more biochemically relevant binding site is likely to be the one closer to the catalytic metal and that the binding behavior has been satisfactorily characterized at physiological pH.

The T_{2p}^{-1} data are also shown in Figures 1–4. As noted above, they are 70 times higher than the corresponding T_{1p}^{-1} values. Such a large difference is difficult to explain. In principle, it may be due to exchange broadening operating on T_2 according to

$$\frac{T_{2p}^{-1}}{f_M} = \tau_M^{-1}\frac{T_{2M}^{-2} + T_{2M}^{-1}\tau_M^{-1} + (\Delta\omega_M)^2}{(T_{2M}^{-1} + \tau_M^{-1})^2 + (\Delta\omega_M)^2} \quad (3)$$

where $\Delta\omega_M$ is the difference in chemical shift between free and protein-bound phosphate. Exchange broadening is maximum when $\tau_M^{-1} = \Delta\omega_M$. In such a case, the experimental T_{2p}^{-1} values are reproduced by eq 3 for $\tau_M^{-1} = \Delta\omega_M = 10^5$ rad s^{-1} (≃500 ppm at 32 MHz). This is a lower limit for $\Delta\omega_M$. Such a value is unrealistically high for phosphate that is not directly coordinated to the copper center. In addition, the phosphate signal would have shown an experimental shift of at least 100 Hz, whereas no shift could be detected in the present case. The temperature and field dependence of the line width should help in clarifying the nature of the T_2^{-1} mechanism. However, the line width turns out to be essentially insensitive to temperature in the range 4–27 °C and to the magnetic field between 2.11 and 7.05 T.

It is surprising that the concentration dependence of T_{2p}^{-1} always parallels, at least qualitatively, that of T_{1p}^{-1}, suggesting that the line broadening is in any case related to the same kind of sites giving rise to detection of paramagnetic effects on T_{1p}^{-1}. There are other ^{31}P NMR studies in which it has been noted that T_{2p}^{-1} is much larger than T_{1p}^{-1} for a phosphate group that is not coordinated to a paramagnetic metal ion.[20] The reasons for such a difference have not been analyzed. Clearly, further efforts should be made to understand this phenomenon, but they are outside the scope of the present work. Whatever the nature of the line broadening is, the conclusions drawn from the T_{1p}^{-1} data should be absolutely reliable for obtaining structural information.[15]

Discussion

The interaction of phosphate with the free amino acid arginine has been demonstrated by observation of small chemical shifts of the ^{31}P resonance near the phosphate pK_{a2} region. This effect was attributed to preferential binding of the dibasic phosphate anion by the guanidinium group of arginine.[21,22] In the present study, the interaction of phosphate with bovine Cu,Zn-SOD was detected by means of the increase in the nuclear relaxation rates of the ^{31}P nucleus due to the proximity of the paramagnetic copper(II) center.[15] Using the measured T_{1p}^{-1} values, we have determined that, at pH 7.0 and 8.0, phosphate binds at a specific site, which is located approximately 5 Å from the copper ion. It is known from the X-ray crystallographic studies[3] that the guanidinium group of Arg-141 is located approximately 5 Å from the copper(II) ion. Previous studies have also demonstrated that modification of Arg-141 with phenylglyoxal diminishes the influence of phosphate on the SOD activity and the anion-binding properties of the protein.[11] These results combined with our

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observation that this modification diminishes the affinity constant for phosphate binding suggest strongly that Arg-141 is the site of phosphate binding.

The concentration dependences of T_{1p}^{-1} parameters for the native protein solutions (Figures 1 and 2) at pH 8.0 and 7.0 yield phosphate affinity constants of 20 ± 4 and 34 ± 3 M^{-1}, respectively. The calculated phosphate-binding constants are thus significantly higher than those previously obtained for the interaction between phosphate and free arginine, i.e., $K = 5$ M^{-1}.[21] This observation suggests that Arg-141 is activated toward phosphate binding, probably as a result of its location in a hydrophobic region of the solvent channel in the protein.

At pH 6.3, two binding constants for phosphate are detected. If we consider that the lower affinity value at pH 6.3 corresponds to the single values observed at pH 7.0 and 8.0, we must conclude that the affinity of this site for phosphate reaches a maximum between pH 6.3 and 8.0. The increase in affinity for phosphate between pH 6.3 and 7.0 is probably due to a higher affinity for HPO_4^{2-} relative to that for $H_2PO_4^-$. The decrease in affinity between pH 7.0 and 8.0 is more difficult to explain. It has been reported that the SOD activity of the protein decreases above pH 7.[23] A possible explanation for both effects is deprotonation of a group other than Arg-141 near the active-site channel. The nature of the second binding site for phosphate at pH 6.3 cannot be identified from the present NMR study.

It is interesting to compare our conclusions with those obtained from studies of phosphate binding to Co,Zn-SOD, a derivative in which Co^{2+} has replaced Cu^{2+} at the native copper site. In this derivative, it has been concluded that phosphate interacts directly with the cobalt chromophore, on the basis of electronic,[24,25] ESR,[24] and ^{31}P NMR[25,26] spectroscopic evidence. On the basis of this evidence, it has been suggested that phosphate binds directly to the cobalt ion and that this direct binding induces the rupture of the imidazolate bridge between cobalt and zinc. These results are thus in sharp contrast to those obtained for the native protein, where phosphate does not appear to bind to copper but is 5 Å away at the guanidinium group of Arg-141. These results are, of course, consistent with the observation of pronounced electronic spectral changes in the visible region upon binding of phosphate to Co,Zn-SOD whereas no visible spectral changes are observed upon binding of phosphate to Cu,Zn-SOD.[11]

Quantitative analysis of phosphate binding to the arginine-modified protein provides evidence that chemical modification of Arg-141 with phenylglyoxal is lowering its affinity for phosphate. The copper–phosphate distance is essentially the same as in the unmodified protein. Chemical modification of an arginine residue with phenylglyoxal[16] places steric hindrance around the arginine side chain without altering its positive charge. We therefore believe that phosphate may still be attracted by the positive charge of the modified arginine residue but that the chemical modification prevents a specific molecular interaction between the side chain and phosphate, resulting in a lower phosphate affinity and, thus, smaller T_{1p}^{-1} values for the arginine-modified protein. Smaller relaxation values cannot be due to the presence of some unmodified native protein present as a contaminant in the preparation of the arginine-modified SOD since the T_{1p}^{-1} profile would only be lowered and not shifted, as is observed.

The functional significance of the binding of phosphate to bovine Cu,Zn-SOD is not immediately apparent to us. Although it is clear from our earlier work[11] that phosphate at concentrations typically present in the cell will cause partial inhibition of the SOD activity of this protein, it is also possible that phosphate may play a role in some as yet unknown physiological function of this protein. Future studies hopefully will clarify the role of phosphate in the function of this protein.

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Registry No. Cu,Zn-SOD, 9054-89-1; Arg, 74-79-3; phosphate, 14265-44-2.

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