accord with this suggestion we have found that dialysis of a 1.0 mM (subunits) solution of Cu_2Zn_2SOD against 0.1 M potassium phosphate at pH 3.6 resulted in removal of 95% of the Zn and 5% of the Cu (by atomic absorption), although the vis-UV and ESR spectra were only slightly changed. Likewise, addition of 1 equiv of Zn^{2+}/subunit to solutions of Cu_2E_2SOD at pH 3.6 caused only small changes in the vis-UV spectra of the solutions, but returning the pH to 6.0 gave spectra identical with those of Cu_2Zn_2SOD. We conclude from these studies either that Zn^{2+} is not bound to the protein at low pH or that it is bound in such a way that its presence does not affect the spectral properties of the Cu(II) chromophore and it is readily removed by dialysis.

Similar results have been obtained for Cu_2Cu_2SOD (see Figures 1 and 2). The ESR experiment at 30 °C is particularly instructive since Cu_2Cu_2SOD is ESR silent at that temperature under our conditions.[18] At low pH, the coppers are uncoupled and the signal due to Cu_2E_2SOD plus the isotropic signal of free aqueous Cu^{2+} appear. The change is almost fully reversed when the pH is raised.

The published vis-UV and ESR spectra of solutions of Cu_2Co_2SOD at low pH[16] also strongly resemble those of Cu_2E_2SOD and free aqueous Co^{2+}. The disappearance of the visible spectrum due to Co^{2+} in an approximately tetrahedral site suggests that Co^{2+} has moved to an octahedral site, such as in $Co(H_2O)_6^{2+}$, where the extinction coefficient of the visible absorption band is expected to be much lower.[19]

The observations described above strongly suggest that the metal ion bound in the native zinc binding site in Cu_2Zn_2SOD, Cu_2Cu_2SOD, or Cu_2Co_2SOD is released in the range $4.5 > pH > 3.0$ and that the metal ion is rapidly re-bound when the pH is raised. The fact that this release of metal ions from the zinc site occurs at very similar pH values whether Zn^{2+}, Cu^{2+}, or Co^{2+} is bound to that site suggests that this phenomenon is not a simple competition between metal ions and protons for the protein side chain ligands. A simple competition reaction would be expected to show a different dependence on pH for the three metal ions if the ligands were conformationally fixed.[20] This observation combined with the observation that E_2Co_2SOD[21] and the apoprotein[14] undergo spectral changes in the same pH range suggest that a pH-dependent conformational change is occurring, the result of which is a lowering of the metal ion affinity of the native zinc site. It is interesting in this regard to note that the protein side chains that comprise the zinc binding site come from a single loop of the polypeptide chain which has a relatively high number of hydrophilic residues in a nonrepetitive secondary structure.[3,4,22] The pH-dependent changes between pH 3 and 4.5 could be accounted for by a conformational change in this loop alone causing only minor changes at the copper binding site.[23]

The pH-dependent nature of the metal ion affinities explains some of the inconsistencies in the early work on metal ion reconstitution of this protein[5] and preparation of metal-substituted derivatives.[10] It also explains the success of the reconstitution procedure of Beem et al.[11,12] in which either Cu^{2+} or Ag^+ is added to apoprotein at pH 3.8 where it binds predominantly at the copper binding site. Our results indicate that the copper binding site is the only strong binding site at that pH.

It is clear from the results described above and previous studies in our laboratory[18] that the metal binding properties of copper-zinc superoxide dismutase are strongly pH dependent. Future studies will be directed toward elucidating the kinetics and thermodynamics of the metal binding reactions of this protein over a wide range of pH.

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References and Notes

(1) Abbreviations: Cu_2Zn_2SOD, native bovine erythrocyte superoxide dismutase; Cu_2M_2SOD (M = Co, Cu) derivative of the native enzyme in which M(II) has been substituted for Zn(II); Cu_2E_2SOD, derivative in which the zinc site is vacant (E = empty); E_2Co_2SOD, derivative in which the copper site is vacant and Co(II) is bound to the zinc site.
(2) R. J. Carrico and H. F. Deutsch, *J. Biol. Chem.*, **245,** 723 (1970).
(3) J. S. Richardson, K. A. Thomas, and D. C. Richardson, *Biochem. Biophys. Res. Commun.*, **63,** 986 (1975).
(4) J. S. Richardson, K. A. Thomas, B. H. Rubin, and D. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.*, **72,** 1349 (1975).
(5) I. Fridovich, *Adv. Enzymol.*, **41,** 35 (1974).
(6) J. A. Fee and B. P. Gaber, *J. Biol. Chem.*, **247,** 60 (1972).
(7) J. A. Fee, *Biochim. Biophys. Acta*, **295,** 107 (1973).
(8) J. A. Fee, *J. Biol. Chem.*, **248,** 4229 (1973).
(9) G. Rotilio, L. Calabrese, B. Mondovì, and W. E. Blumberg, *J. Biol. Chem.*, **249,** 3157 (1974).
(10) For reviews of metal substitutions in Cu_2Zn_2SOD, see J. A. Fee (p 173) and G. Rotilio and L. Calabrese (p 193) in "Superoxide and Superoxide Dismutases", A. M. Michelson, J. M. McCord, and I. Fridovich, Eds., Academic Press, London, 1977.
(11) K. M. Beem, D. C. Richardson, and K. V. Rajagopalan, *Biochemistry*, **16,** 1930 (1977).
(12) K. M. Beem, W. E. Rich, and K. V. Rajagopalan, *J. Biol. Chem.*, **249,** 7298 (1974).
(13) J. A. Fee and R. G. Briggs, *Biochim. Biophys. Acta*, **400,** 439 (1975).
(14) J. A. Fee and W. D. Phillips, *Biochim. Biophys. Acta*, **412,** 26 (1975).
(15) G. Rotilio, L. Morpurgo, L. Calabrese, A. Finazzi-Agrò, and B. Mondovì in "Metal-Ligand Interactions in Organic Chemistry and Biochemistry", B. Pullman and N. Goldblum, Eds., Part 1, D. Reidel, Dordrecht, Holland, 1977, p 243.
(16) L. Calabrese, D. Cocco, L. Morpurgo, B. Mondovì, and G. Rotilio, *FEBS Lett.*, **59,** 29 (1975).
(17) S. J. Lippard, A. R. Burger, K. Ugurbil, M. W. Pantoliano, and J. S. Valentine, *Biochemistry*, **16,** 1136 (1977).
(18) J. S. Valentine, M. W. Pantoliano, P. J. McDonnell, A. R. Burger, and S. J. Lippard, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
(19) F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry", 3rd ed., Wiley, New York, 1972, p 881.
(20) H. Sigel and D. B. McCormick, *Acc. Chem. Res.*, **3,** 201 (1970).
(21) L. Calabrese, D. Cocco, L. Morpurgo, B. Mondovì, and G. Rotilio, *Eur. J. Biochem.*, **64,** 465 (1976).
(22) D. C. Richardson in "Superoxide and Superoxide Dismutases", A. M. Michelson, J. M. McCord, and I. Fridovich, Eds., Academic Press, London, 1977, p 217.
(23) It should be noted that a reorganization of the ligands that comprise the metal binding region may have occurred at low pH resulting in the existence of only one strong metal binding site, with a higher affinity for Cu^{2+} than for Zn^{2+} or Co^{2+}. In other words, we cannot be certain that the same four histidyl side chains are bound to Cu^{2+} at low pH as are bound at high pH.
(24) ESR spectra were recorded using an aqueous ESR cell (Wilmad) at ambient temperature (30 ± 2 °C) together with a Varian E-12 spectrometer at 100-kHz field modulation and a sweep rate of 180 G/min. The Cu_2Zn_2SOD and Cu_2E_2SOD spectra were obtained using a microwave power of 30 mW at a frequency of 9.41 GHz and a modulation amplitude of 8.0 G. The gain was 2500 while for Cu_2Cu_2SOD it was increased by a factor of 2.5 and the modulation amplitude was increased to 10.0 G. The magnetic field was calibrated using the signal of Mn(II) naturally present as an impurity in SrO.[18] Microwave frequency measurements were made using a Hewlett-Packard Model 5255A frequency meter. All ESR spectral parameters are defined according to B. Malmstrom and A. Vänngård, *J. Mol. Biol.*, **2,** 118 (1960).

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P-450 Type Oxygen Activation by Porphyrin-Manganese Complex

Sir:

Since cytochrome P-450 was first isolated in 1955,[1] much attention has been given to structural[2] and/or mechanistic[3] elucidation of this interesting and somewhat unique enzyme.

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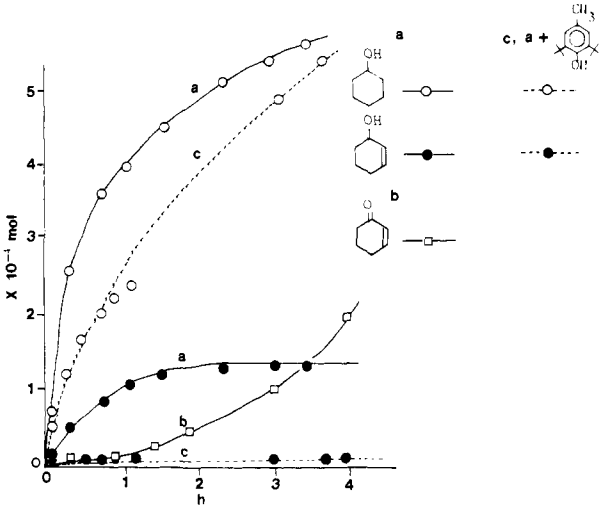


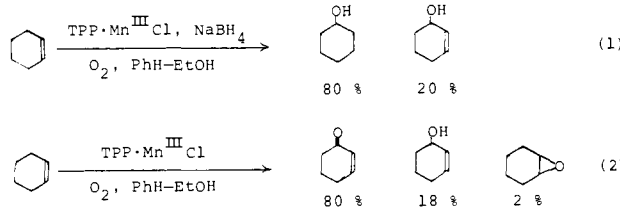
Figure 1. Time-conversion curve of the oxygenation with TPP·Mn-O_2: a, with $NaBH_4$; b, without $NaBH_4$.

The major structural interest has been concentrated on the electronic state involving the spin state of central metal ion, iron, which is closely connected to the major mechanistic interest in why P-450 can *activate* a dioxygen molecule while such an oxygen carrier protein of a very similar local structure as myoglobin or hemoglobin forms a stable dioxygen complex. The mechanistic difference between them is, however, very delicate in a sense that, even for the oxygen carrier protein, a considerable electron transfer from ferrous to the bound dioxygen is expected,[4] which is taken as one of the characteristics of P-450 mechanism, although its function is quite different.

Axial ligation of sulfur of cystein which is not present in the oxygen carrier protein is considered a crucial factor for oxygen activation by P-450, but any other factor, such as efficient reduction of P-450 owing to its appropriate size to accommodate NADH near its active site, may also be responsible for oxygen activation.

Now, we report herein that manganese(II) porphyrin in the presence of $NaBH_4$ can activate a dioxygen molecule efficiently even in the absence of such an assisting ligand as SH, affording an excellent P-450 model system. Thus, in the presence of a small amount of tetraphenylporphyrin·$Mn^{III}Cl$ (9.3×10^{-3} M) and an excess amount of $NaBH_4$, cyclohexene was treated with an excess amount of an oxygen molecule (air) in benzene–ethanol at room temperature. The rapid reaction took place giving cyclohexanol and cyclohexenol in a 4:1 ratio without formation of any appreciable byproducts.

The three components of this reaction, TPP·Mn, $NaBH_4$, and O_2, are essential, closely corresponding to the three essential components in the P-450 oxygenation, protein·Fe, NADH, and O_2. In the absence of $NaBH_4$, cyclohexene was



also oxidized to give cyclohexenone and other products as shown in eq 2, but a remarkable *induction period* was observed as shown in Figure 1, and the oxidation was almost perfectly quenched by the addition of such a typical radical inhibitor as 2,6-di-*tert*-butylphenol. These facts clearly indicate that, in the absence of $NaBH_4$, the reaction proceeds as a typical autoxidation. However, in the presence of $NaBH_4$ the TPP·Mn–$NaBH_4$–O_2 reaction proceeds in a quite different way, showing the following significant characteristics: (i) its striking selectivity to give cyclohexanol as shown in eq 1, (ii) the remarkable acceleration as is apparent in Figure 1, (iii) absence of any induction period in the cyclohexanol formation (Figure 1), and (iv) absence of inhibitory effect of a typical chain inhibitor, 2,6-di-*tert*-butyl-*p*-cresol, on cyclohexanol formation (see Figure 1).

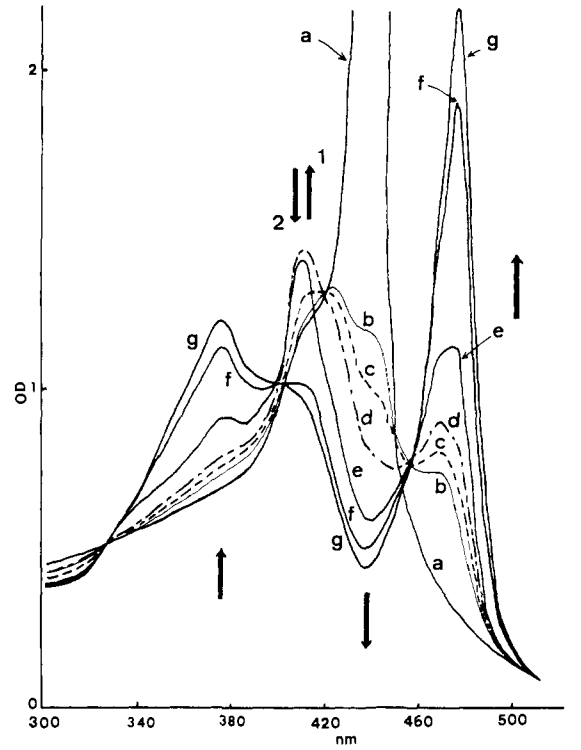
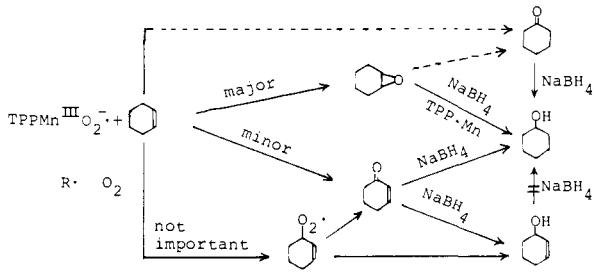


Figure 2. Electronic spectra of TPP·Mn and intermediates derived from TPP·Mn(II) + O_2: a, TPP·Mn(II); b, TPP·Mn(II) + O_2, after 20 s (—); c, after 80 s (- - -); d, after 200 s (- - -); e, after 260 s; f, after 1 h; g, TPP·Mn(III).

Among the most probable origins of cyclohexanol in the TPP·Mn–$NaBH_4$–O_2 reaction, cyclohexenol, which may be formed from cyclohexene directly, should be excluded since independent experiments showed that, under the present conditions, cyclohexenol was not appreciably reduced to cyclohexanol. Further oxygenation of cyclohexenol was only within experimental error in experiments using cyclohexenol (0.018 M) and cyclohexene (3.3 M) as a starting system.[5] Cyclohexenone was readily reduced under the condition, giving cyclohexenol and cyclohexanol in a ratio of 1.4:1, indicating that it can not be principal origin of, but only participates in, the formation of cyclohexanol in 14% yield ($20 \times 1/1.4$) at most.[6] The significant contribution of the allylic oxidation is also excluded based on the results of the 1-hexene oxidation where 2- and 1-hexanol were obtained as principal products in a ratio of 5.3–6.4:1 after 3 to 5 h; the 3-hexanol formation was <1% of the 2-hexanol formation. These results also successfully eliminate the possibility of the significant contribution of hydroboration (with and/or without TPP·Mn catalysis) from which the principal formation of 1-hexanol should be expected. The principal origin (86% at least) should be another source. The remaining candidate, cyclohexene oxide, seems to be the most probable based on the following observations: (a) cyclohexene oxide was actually formed under the condition of TPP·$Mn^{III}Cl$–$NaBH_4$–O_2 in benzene even in the presence of a large excess of $NaBH_4$; (b) cyclohexene oxide was very readily reduced with TPP·$Mn^{III}Cl$ + $NaBH_4$ to cyclohexanol exclusively.

Thus, the most probable pathway of the present reaction is

Scheme I. Pathway of the TPP·Mn–O_2–$NaBH_4$ (P-450 Type) Oxidation



that shown in Scheme I. Under conditions where cyclohexene was converted into oxygenated products in 2.5% yield,[7] yields of cyclohexanol and cyclohexenol based on O_2 used were 53 and 13%, respectively, and that based on TPP·Mn was 1530% (each TPP·Mn molecule produced 15.3 molecules of oxygenated products). Most of the $NaBH_4$ used in an excess amount was recovered unchanged after the oxygenation was over.

Pure TPP·$Mn^{III}Cl$ treated with $NaBH_4$ in carefully purified benzene showed the characteristic electronic spectrum of TPP·Mn^{II} as shown in Figure 2. Application of dioxygen to the solution finally resulted in practically complete conversion into the corresponding Mn(III) (see Figure 2). However, neither of the individual final products, TPP·Mn^{III} nor $O_2^{-}\cdot$, gave the observed oxidation products. While a mixture of both components TPP·Mn^{III} and KO_2 in the presence of 18-crown-6 gave the oxygenation products, the yields were low. Thus, the active species should be some intermediate between the two extreme states of eq 3.

$$\text{TPP·Mn(II)} + O_2 \longrightarrow [\text{intermediate}] \longrightarrow \text{TPP·Mn(III)} + O_2^{-} \quad (3)$$
$$\downarrow \text{substrate}$$
$$\text{oxygenation products}$$

Careful spectroscopic investigations indicated that at least two intermediates (Mn^{IV}·O_2^{2-} (side-on complex[8]) and Mn^{III}–O_2^{-} complex) were involved in the reaction. Thus, the fact that the reducing reagent, $NaBH_4$, and one of these intermediates, possibly TPP·Mn^{III}–OO^{-}, seem to provide the real active species the fact again closely resembles the established cyt-P-450 mechanism.

Detailed studies are now underway.

References and Notes

(1) Klingenberg, M. *Arch. Biochem. Biophys.* **1958**, *75*, 376.
(2) (a) Peterson, J. A. *Arch. Biochem. Biophys.* **1971**, *144*, 678. (b) Dawson, J. H.; Cramer, S. P. *FEBS Lett.* **1978**, *88*, 127. (c) Sharrock, M.; Derumner, P. G.; Schulz, C.; Lipscomb, J. D.; Marshall, V.; Gunsalus, I. C. *Biochim. Biophys. Acta* **1976**, *420*, 8. (d) Champion, P. M.; Gunsalus, I. C.; Wagner, G. C. *J. Am. Chem. Soc.* **1978**, *100*, 3743. (e) Tang, S. C.; Koch, S.; Papaefthymiou, G. C.; Foner, S.; Frankel, R. B.; Ibers, J. A.; Holm, R. H. *Ibid.* **1976**, *98*, 2418.
(3) (a) Sato, R.; Omura, T. In "Cytochrome P-450"; Kodansha Ltd: Tokyo, 1978. (b) Boyd, G. S.; Ullrich, V. *Biochem. Soc. Trans.* **1975**, 803. (c) Nordblom, G. D.; White, R. E.; Coon, M. J. *Arch. Biochem. Biophys.* **1976**, *175*, 524.
(4) Calculation on iron(II) porphyrin·O_2 Im (as HRP compound I model) and iron(II) porphyrin·O_2 S^{-}–Me (as P-450 model). Loew, G. H.; Kert, C. J.; Hjelmeland, L. M.; Kirchner, R. F. *J. Am. Chem. Soc.* **1977**, *99*, 3534.
(5) (a) TPP = tetraphenylporphyrin. (b) It is simply a concentration problem since initial concentration of cyclohexene was much larger than that of the product, cyclohexenol.
(6) Since the major path of cyclohexenol formation seems to be direct oxidation, this value is a considerable overestimation.
(7) Conversion was kept low in order to suppress any undesirable further oxidation.
(8) Hoffman, B. M.; Weschler, C. J.; Basolo, F. *J. Am. Chem. Soc.* **1976**, *98*, 5473.

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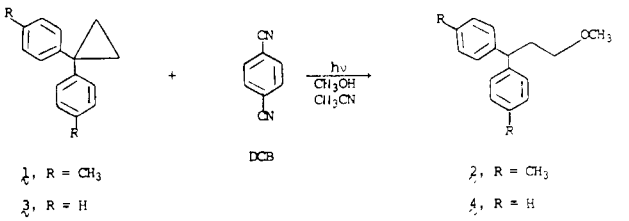
Arylcyclopropane Photochemistry. Electron-Transfer-Mediated Photochemical Addition of Methanol to Arylcyclopropanes

Sir:

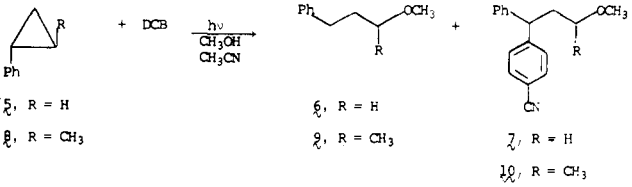
The photosensitized cis–trans isomerization of 1,2-diphenylcyclopropane with naphthalene derivatives as sensitizers has been suggested to proceed via formation of a singlet exciplex.[1] It has been shown that charge transfer from the cyclopropane to the excited singlet naphthalene sensitizer is important in formation and/or reaction of this exciplex.[2] We presently report the 1,4-dicyanobenzene (DCB)-sensitized photolysis of several phenylcyclopropane derivatives in methanol–acetonitrile. In most cases a novel "anti-Markownikoff" addition of methanol to the cyclopropane ring results. The reactions observed are best rationalized as proceeding via initial electron transfer from the cyclopropanes to the excited DCB to give the phenylcyclopropane radical cations.[3]

Several phenylcyclopropanes have been studied to date. In a typical reaction a solution of 1.4 mmol of 1,1-bis(*p*-tolyl)-cyclopropane (**1**), 0.7 mmol of DCB, and 1.0 mL of methanol in 12.0 mL of acetonitrile in a Vycor reaction vessel was purged with nitrogen and then irradiated at 45 °C with the 300-nm lamps of a Rayonet photochemical reactor. Progress of the reaction was monitored by gas chromatography. After 10 h (70% reaction of **1**), the solvent was removed and the photolysate was separated by preparative TLC (silica gel plates with benzene eluant) to give 3-methoxy-1,1-bis(*p*-tolyl)propane[4] (**2**, 71%) together with recovered cyclopropane **1** (7%) and



DCB (88%). No other products were detected by GC, TLC, or NMR analysis of the reaction mixture. 1,1-Diphenylcyclopropane (**3**) reacted similarly to give ether **4** (30% yield at 92% conversion of **3**).

Phenylcyclopropane (**5**) and 1-methyl-2-phenylcyclopropane (**8**) afforded similar methanol adducts; however, in both cases a new product was isolated. Thus, DCB-sensitized photolysis of **5** gave 41% ether **6** and, in addition, 17% diaryl product **7** (84% conversion of **5**). Likewise, *trans*-**8** (containing ~3% of the cis isomer) yielded, at 85% conversion of **8**, 32%



ether **9** and 16% **10**. Interestingly, little, if any, isomerization of *trans*-**8** to its cis isomer occurred during the photolysis.

The DCB-sensitized photolysis of *trans*-1-methyl-2-phenylcyclopropane (**8**) in acetonitrile in the absence of methanol afforded no detectable new products. Slow loss of the reactants was noted, as well as a very small amount (~1%) of trans to cis isomerization.[5] (Photolysis of **8** in the absence of DCB under otherwise similar reaction conditions resulted in more efficient (but still slow) trans–cis isomerization.)[6]

The sensitized photolysis of 1,1,2,2-tetraphenylcyclopropane (**11**) took a different course. Only traces of a methanol adduct (presumably **12**) could be detected by NMR and GC.

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