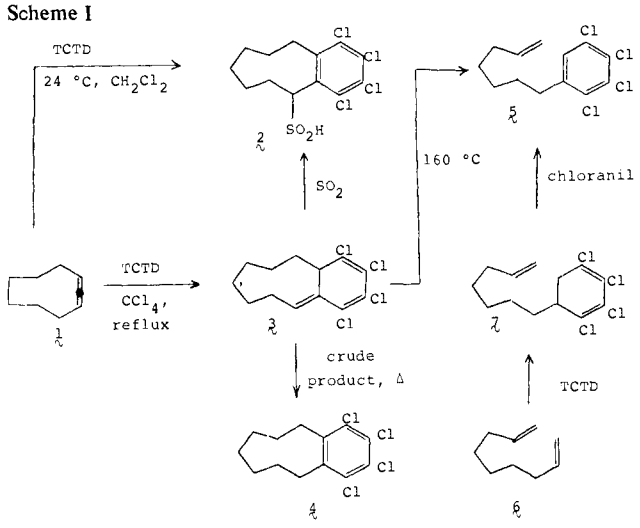
Scheme I



yield). Thus, the two steps by which **2** is formed at 24 °C are apparent.

The successful isolation of the sulfinic acid **2** from the ene reaction of **3** with sulfur dioxide is direct evidence that supports the initial step in the isomerization mechanism proposed by Rogić and Masilamani. The sulfinic acid is isolable in this case because the benzene ring does not participate in the rearrangement and retro-ene reactions characteristic of allylic sulfinic acids.[1b]

If a crude sample of **3** containing a small amount of **2** is distilled under vacuum, it undergoes an acid-catalyzed double-bond migration to the thermodynamically more stable benzocyclononene **4** (mp 110.5–111.7 °C). On the other hand, if pure **3** is heated at 160 °C, it undergoes an unusually facile, nearly quantitative retro-ene reaction to give **5** (mp 30.3–30.9 °C, bp 103 °C (3 mm)). The preference for a thermal [1, 5] shift over the symmetry forbidden [1, 3] sigmatropic shift in this system is evident. The number of known examples of this retro-ene reaction in all carbon systems is small, and they require temperatures in the range of 250–580 °C.[5] For example, *cis*-cyclononene itself is converted into 1,8-nonadiene to the extent of only 20% at 500 °C.[6] The more facile rearrangement in the present case can be attributed to the formation of the aromatic ring.

The preparation of **3**, uncomplicated by the presence of sulfur dioxide, also was carried out by heating 1,2-cyclononadiene with tetrachloro-α-pyrone[7] at 100 °C. The structure of **5** was corroborated by independent synthesis (Scheme I). 1,8-Nonadiene[8] (**6**) was monoannelated with TCTD at ~100 °C to give **7** (bp 131 °C (0.5 mm)) in 49% isolated yield. Compound **7** was aromatized to **5** by heating with chloranil at 160 °C.

Acknowledgment. We are indebted to F. A. Blissick for technical assistance and to E. W. Matthews for IR and UV spectral interpretations.

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^{113}Cd NMR of Metallothionein: Direct Evidence for the Existence of Polynuclear Metal Binding Sites

Sir:

The structural basis for the remarkable metal binding capacity of metallothionein (~6–7 g-atoms/mol, usually Zn^{2+} and Cd^{2+}) is of considerable interest owing to the recent postulated involvement of this protein in metal detoxification and/or metabolism.[1a,2] Metallothionein is a small protein (mol wt, ~6800) located in the kidney and liver of a wide variety of animal species, including man, and its synthesis can be induced by the administration of Cd^{2+}, Zn^{2+}, and other heavy metals.[3] The amino acid composition of the protein is very unusual in that there is a complete absence of histidine and aromatic amino acid residues while about 20 out of the total of 60 amino acids in the protein are cysteine residues.[1a,2c] All 20 cysteines have been shown to participate in metal ligation via mercaptide linkages.[1a,4] Since the ratio of cysteine/divalent metal ion in all metallothioneins is close to 3, it has been postulated that the metals in the protein exist as isolated, negatively charged trimercaptide complexes,[5] i.e., $[Me^{2+}(Cys^-)_3]^-$. In view of the known propensity of Cd^{2+} to form polynuclear complexes deriving from the ability of mercaptide sulfur atoms to act as bridges between two or three Cd^{2+} ions,[6] alternate structures involving oligomeric metal binding sites are equally likely to exist in metallothionein. In this paper, we report the observation of extensive ^{113}Cd–^{113}Cd spin coupling in the ^{113}Cd NMR spectrum of metallothionein II from rabbit liver. This result provides the first direct evidence for the existence of a polynuclear metal cluster arrangement in this protein.

Pure metallothionein II (one of the major isoproteins of metallothionein) was isolated[1a] from the livers of rabbits subjected to repeated injections of 96% enriched $^{113}CdCl_2$. Protein was concentrated for NMR to ~14 mM by ultrafiltration on a Diaflo UM-2 membrane. The metal content of the protein determined by atomic absorption and normalized to a total of 7 g-atoms of metal/mol of protein was 4.4 g-atoms of $^{113}Cd^{2+}$ and 2.6 g-atoms of Zn^{2+}/mol. The nonintegral metal content reflects the known heterogeneity of the native protein which is due to the presence of species containing different relative amounts of Zn^{2+} and Cd^{2+}.[3] ^{113}Cd spectra at 19.96 MHz were obtained on an extensively modified Bruker HFX-90 spectrometer and at 44.37 MHz on a Bruker CXP-200 spectrometer.

The ^{113}Cd NMR spectrum of metallothionein II in Figure 1A exhibits five resolved resonances centered at 604, 613, 623, 641, and 668 ppm downfield from 0.1 M $CdClO_4$. These chemical shift values are consistent with previous reports[7] and indicate that each Cd^{2+} ion in metallothionein is involved in extensive sulfur ligation. From the qualitative correlation between degree of deshielding and extent of sulfur ligation observed in a series of Cd^{2+}-alkylthiolate complexes,[8] it is likely that every Cd^{2+} ion in metallothionein is coordinated to at least three cysteine sulfur ligands. However, on the basis of available

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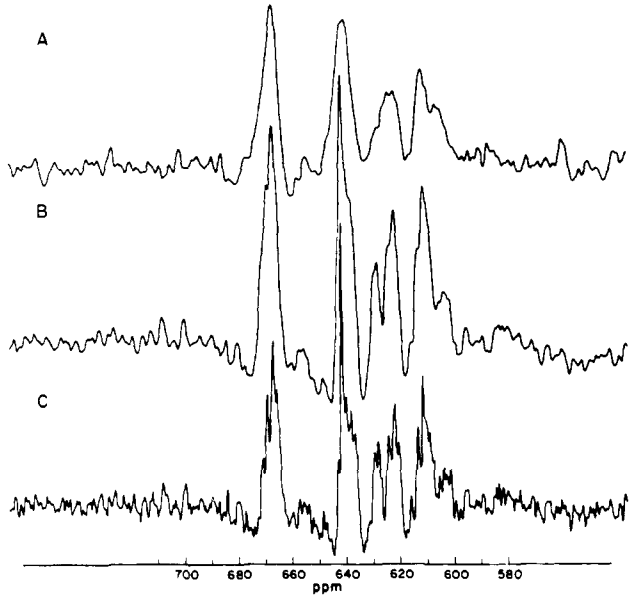

Figure 1. ^{113}Cd NMR spectra at 19.96 MHz of rabbit liver metallothionein II in 0.01 M Tris, 0.1 M NaCl, pH 8.8 (the protein concentration was ~14 mM in 1.25 mL): A, coupled spectrum (7500 transients, 32-Hz digital broadening); B, gated proton-decoupled spectrum (10 000 transients, 32-Hz digital broadening); C, same spectrum as B, plotted with 11-Hz digital broadening. All spectra were acquired using a 90° pulse and 8-s recycle time.

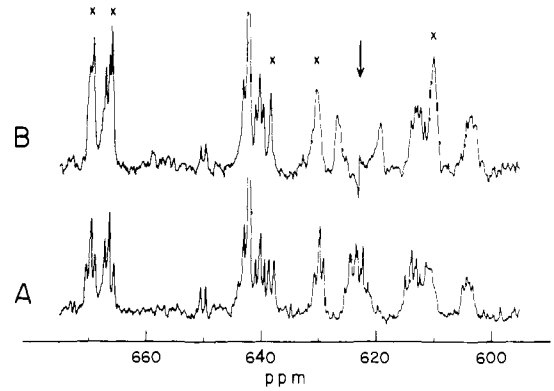

Figure 2. ^{113}Cd NMR spectra at 44.37 MHz of rabbit liver metallothionein II in 0.01 M Tris, 0.1 M NaCl, pH 8.8 (the protein concentration was ~14 mM in 2.1 mL): A, proton-decoupled spectrum (7200 transients, 8-Hz digital broadening); B, same as A but with application of a selective homonuclear decoupling pulse at the frequency indicated by the arrow. Resonances undergoing partial or complete collapse of coupling are identified with an x. Spectra were acquired using a 70° pulse and 1.6-s recycle time.

chemical shift data, it is not possible to distinguish between metallothionein metal binding sites consisting of three nonbridging cysteine ligands and sites containing four or more sulfur ligands, one or more of which bridge adjacent metal ions.

The spectrum in Figure 1B demonstrates the significant improvement in spectral resolution which accompanies proton decoupling owing to the removal of unresolved splitting presumably originating from the three-bond coupling of the $^{113}Cd^{2+}$ ions to the β protons on the cysteine ligands.[9] The difference between the coupled and decoupled spectra is most easily seen by the resolution of the broad resonance at 623 ppm in Figure 1A into two peaks at 630 and 623 ppm in Figure 1B. However, the most striking effect of proton decoupling is seen when the spectrum is replotted with less digital broadening (Figure 1C). With the proton coupling removed, it becomes possible to resolve considerable underlying fine structure in each of the ^{113}Cd resonances. The observed splittings must arise either from small chemical shift differences, possibly resulting from the metal ion heterogeneity of the native protein sample, or from spin–spin coupling, which must be ^{113}Cd–^{113}Cd coupling since there are no other abundant magnetic nuclei besides protons in the protein. The most direct way to differentiate between these two possibilities is to examine the magnetic field dependence of the fine structure, since only spin couplings will be independent of the applied field. We have therefore acquired the ^{113}Cd NMR spectrum of metallothionein II at 2.2 times higher field strength (Figure 2A). Comparison of the magnitude of the splittings in Figures 1C and 2A indicates that most of the fine structure is field independent and must therefore arise from ^{113}Cd–^{113}Cd spin coupling. Unequivocal confirmation of the origin of the splitting has been provided by homonuclear decoupling experiments. An example is shown in Figure 2B where a decoupling pulse applied at the center of the complex multiplet at 623 ppm produces a collapse or partial collapse of several other multiplets in the spectrum (indicated by an x).

By analogy to known Cd^{2+}–thiolate polynuclear complexes,[6] the origin of the spin coupling must be a two-bond interaction between adjacent $^{113}Cd^{2+}$ ions mediated by one or more bridging cysteine sulfur ligands. This result therefore provides the first evidence for a polynuclear metal cluster arrangement in metallothionein. Furthermore, to our knowledge this represents the only known example of ^{113}Cd–^{113}Cd spin coupling, although two-bond metal–metal couplings have been reported for ^{195}Pt complexes.[12] The ability to detect ^{113}Cd–^{1}H and ^{113}Cd–^{113}Cd spin coupling in metallothionein is a direct result of the stability of the metal–protein complex. Analogous couplings in smaller $^{113}Cd^{2+}$–alkylthiolate complexes in solution have not been observed,[8] presumably because of chemical exchange averaging resulting from ligand exchange processes which are fast compared with $1/J$.

Several additional aspects of the ^{113}Cd spectra of metallothionein are pertinent in terms of providing information about the structure of this protein. First, although most of the splittings in Figure 1C arises from ^{113}Cd–^{113}Cd coupling, some are clearly the result of chemical shift differences. This is best seen in the case of the most downfield resonance at 668 ppm in Figure 1C, which at the higher field is clearly separated into two peaks, each of which has residual splitting due to ^{113}Cd–^{113}Cd coupling (Figure 2A). In the high-field spectrum it is apparent that $^{113}Cd^{2+}$ is located in more than seven chemically distinct environments, despite the fact that metallothionein is thought to contain only seven metal binding sites. This observation is consisted with the known heterogeneity of the native protein with respect to relative metal-ion composition. By employing a combination of selective homonuclear decoupling and spin echo techniques, we expect in the near future to arrive at a complete assignment of the origin of each of the multiplets present in Figure 2A. This information will thereby provide the means of determining both the extent of and structural basis for the observed heterogeneity of native metallothionein. Finally, it is observed that the field-independent splittings in the ^{113}Cd metallothionein spectra vary from ~30 to 50 Hz. This range may in part be due to the non-first-order nature of some of the ^{113}Cd–^{113}Cd splittings or to possible differences in bonding character in the bridging Cd^{2+}–thiolate bonds arising from the capability of mercaptide sulfur to bridge either two or three Cd^{2+} ions. The existence of any branching in the metallothionein metal cluster(s) will also become apparent following assignment of the multiplets in Figure 2A.

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stitute of Environmental Health Science, NIH Grant AM 18778, and NSF Grant PCM77-18941.

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Radicals of Isobacteriochlorins: Models of Siroheme and Sirohydrochlorin

Sir:

The biological assimilation of sulfite and nitrite is enzymatically mediated by sulfite and nitrite reductases which catalyze the six-electron reductions of sulfite to hydrogen sulfide and of nitrite to ammonia:

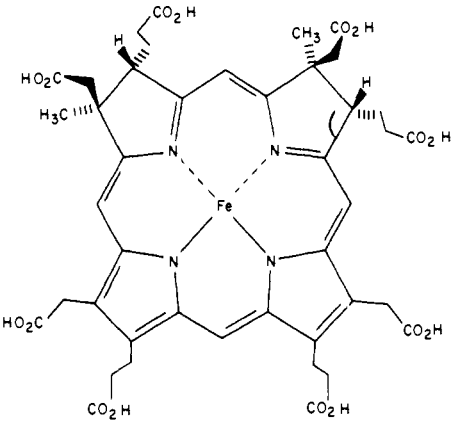
$$SO_3^{2-} + 8H^+ + 6e^- \rightarrow H_2S + 3H_2O,$$
$$\text{and } NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$$

In green plants, the latter reaction is light driven. Photosynthetically reduced pyridine dinucleotide serves as an electron source for the reduction of the enzyme:

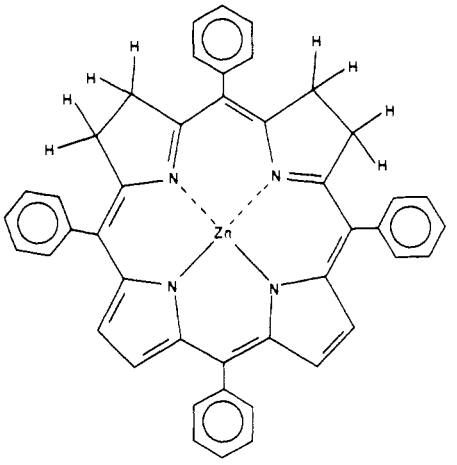
$$\text{chlorophyll} \xrightarrow{h\nu} \text{NADH} \rightarrow \text{flavoprotein} \rightarrow \text{ferrodoxin} \rightarrow \text{nitrite reductase}$$

The prosthetic group of the enzymes has recently been shown[1] to contain siroheme, an iron isobacteriochlorin with eight carboxylic acid side chains (I). A further biological role for isobacteriochlorins has also been invoked[2] with the identification of sirohydrochlorin, a demetalated siroheme, as an intermediate in the biosynthesis of vitamin B_{12}.

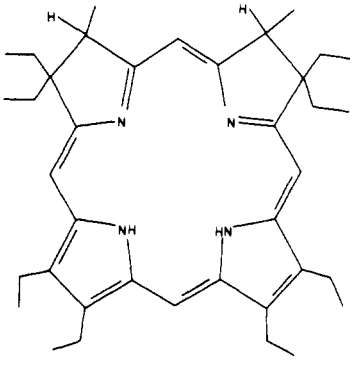
A salient feature of the isobacteriochlorin skeleton is its ease of oxidation and difficulty of reduction compared with those of porphyrins or chlorins.[3] In particular, the facile oxidation (vide infra), raises the intriguing possibility that the ligand itself may undergo redox reactions in the multielectron reductions of the substrates to ammonia and hydrogen sulfide. (Examples of biological two-electron transfers which involve several oxidation states of iron and electron abstraction from the porphyrin are found[4] in catalase and horse radish peroxidase, enzymes which contain heme prosthetic groups.)



I



II



III

To probe the magnetic, optical, and redox characteristics of the isobacteriochlorin class, we describe here MO calculations and ESR, ENDOR, optical, and electrochemical results for two model compounds: zinc tetraphenyl isobacteriochlorin (ZnTPiBC, II), whose recently determined[3b] X-ray structure verifies that two adjacent pyrrole rings are reduced in isobacteriochlorins, and dimethyloctaethyl isobacteriochlorin (H_2DMeOEiBC, III), a compound[3d] which is a close analogue, structurally and spectrally, to sirohydrochlorin.

Electrochemical oxidation[5] of H_2DMeOEiBC in CH_2Cl_2 at +0.5 V (vs. SCE) requires 1 (±0.1) electron to yield the optical spectrum shown in Figure 1. One-electron reduction at 0.0 V regenerates better than 95% of the original H_2DMeOEiBC spectrum. Oxidation to the cation induces a bleaching of the 600-nm band, the appearance of a weak broad

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