

2.8: Metal Substitution

With zinc enzymes, metal substitution is a convenient tool for monitoring the protein and its function by means of spectroscopic techniques. Furthermore, it is interesting to learn how reactivity depends on the nature of the metal ion and its coordination properties, because much of it depends on the protein structure, which seemingly remains constant. As discussed, zinc enzymes can be studied by replacing zinc with other spectroscopically useful metal ions, whose activities have been checked, and by transferring the information obtained to the native enzyme. The strategy of metal substitution is not limited to zinc enzymes, since it has been used for magnesium-activated enzymes and, occasionally, other metalloenzymes as well.

By dialyzing a protein solution against chelating agents, such as EDTA, 1,10-phenanthroline, or 2,6-dipicolinic acid at moderately acidic pH, or by reversibly unfolding the protein with denaturing agents (as has been done with alkaline phosphatase), one can cause zinc proteins to release their metal ions, giving rise to the corresponding but inactive apoprotein. Sometimes (e.g., by using alcohol dehydrogenase) dialysis against chelating agents can be applied to a suspension of protein microcrystals. In this way the chelating agent is still able to reach and remove the active site metal by slowly diffusing in the crystals through the hydration water, while the apoprotein is maintained in the native conformation by the crystal packing forces and denaturation is avoided. After the chelating agent is dialyzed out, often against a high-salt (e.g., ClO_4^-) buffer to reduce nonspecific binding, a new metalloprotein can be obtained by addition of the appropriate metal salt.²³

Cobalt(II)-substituted zinc proteins often show about as much activity as the native zinc enzymes (Table 2.4). This is a general characteristic of the cobalt-substituted zinc enzymes,²⁴ since the coordination chemistry of cobalt(II) is very similar to that of zinc(II). The two ions also show virtually identical ionic radii. Cobalt(II) derivatives generally display useful electronic spectra. High-spin cobalt(II) ions are paramagnetic, containing three unpaired electrons ($S = \frac{3}{2}$); thus they can also give rise to EPR spectra. The electronic relaxation times, i.e., the average lifetimes of the unpaired electrons in a given spin state of the S manifold ($-\frac{3}{2}$, $-\frac{1}{2}$, $\frac{1}{2}$, $\frac{3}{2}$), are very short (10^{-11} to 10^{-12} s) at room temperature. In order to detect EPR spectra, the sample temperature is usually decreased, often down to liquid helium temperature, to increase the electronic relaxation times and sharpen the EPR linewidths. On the other hand, as the paramagnetic broadening of the NMR lines in such systems is inversely proportional to the electronic relaxation times (see Section IV.C.3), room-temperature ^1H NMR spectra of cobalt(II) complexes can be easily detected, even in the absence of chemical exchange. Therefore cobalt(II) is an exceptional probe to monitor the structure and reactivity of zinc enzymes. Of course, the transfer of information from the artificial to the native enzyme must be done with caution. However, if we can understand the functioning of the cobalt enzyme, we then have a reference frame by which to understand the kinetic properties of the native enzyme. The spectroscopic properties of cobalt(II) in cobalt-substituted proteins have been reviewed.²⁵

Copper(II)-substituted zinc proteins are generally inactive with respect to the natural and most artificial substrates (Table 2.4). In model compounds copper(II) is often principally four-coordinate, with at most two more ligands present at metal-ligand distances that are longer than normal coordination bonds. As a consequence, the ability of zinc to switch between four- and five-coordinate species without any appreciable barrier and with usual metal-donor distances is not mimicked by copper. Furthermore, binding at the four principal coordination positions is generally stronger for copper than for zinc. It follows that substrates may have slow detachment kinetics. These properties are unfavorable for catalysis.

Copper(II) can be easily and meaningfully studied by means of electronic spectroscopy. Moreover, the EPR spectra can be recorded even at room temperature because of the long electronic relaxation times, which are of the order of 10^{-9} s. Because a protein is a macromolecule, it rotates slowly, and the EPR spectra in solution at room temperature look like those of crystalline powders or frozen solutions (powder-like spectra). ENDOR spectra are also easily obtained for copper proteins at low temperatures, because at low temperature the electronic relaxation times are even longer, and saturation of the EPR lines (which is a requirement to obtain ENDOR spectra) is easy to accomplish. The long electronic relaxation times make the broadening effects on the NMR lines of nuclei sensing the metal ion too severe; so these lines, unlike those of cobalt(II) complexes, generally escape detection. However, if the nucleus under investigation is in fast exchange between a free species in large excess and a bound species, the line may be observed, because the broadening effects are scaled down by a factor equal to the molar fraction of bound species. The nuclear relaxation parameters contain precious structural and/or dynamic information (see Section IV.C.3). The spectroscopic properties of copper(II) in proteins have been extensively reviewed.^{26,27}

Cadmium-substituted zinc proteins may also be active (Table 2.4), although usually at higher pH. This observation is readily explained in terms of the pK_a of a coordinated water, which is expected to be higher than that of analogous zinc complexes because the cadmium ion is larger and polarizes the Cd-OH_2 bond less.

^{113}Cd and ^{111}Cd are nuclei with relatively high sensitivity for NMR spectroscopic study. The ^{113}Cd chemical shift spans from -200 to 800 ppm relative to CdSO_4 in H_2O , depending on the number and nature of donor atoms.^{24,28} Sulfur donor atoms cause larger downfield shifts than oxygens or nitrogens, and the downfield shift increases with decreasing number of donor atoms. Therefore, ^{113}Cd NMR probes have been used extensively to study zinc enzymes, metal-storage proteins like thioneins, and other proteins with cysteine ligands, and chemical shifts in various cadmium proteins, together with the proposed ligand donor set, have been obtained (Figure 2.1).

Manganese(II)-containing proteins give rise to detectable EPR signals; however, their interpretation in terms of structure and dynamics is not always informative. The electronic relaxation times of Mn^{2+} are the longest among metal ions, of the order of 10^{-8} s at room temperature and at the magnetic fields of interest. This property and the large $S = \frac{5}{2}$ value account for a large NMR linewidth, even larger than in copper(II) systems. Manganese(II)- and nickel(II)- substituted zinc proteins have often been reported to have fractional activity (Table 2.4).²⁴ Several efforts have been devoted to Mn(II) derivatives, especially by studying the NMR signals of nuclei in molecules that exchange rapidly with the metalloprotein.

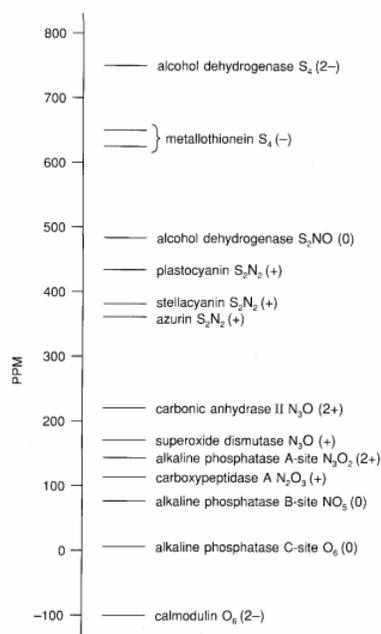


Figure 2.1 - ^{113}Cd chemical shifts in cadmium(II)-substituted metalloproteins.^{24,28} Donor sets and overall charges (in parentheses) of the complexes are indicated.

Finally, several other metal-substituted zinc metalloprotein derivatives have been prepared, including those of VO^{2+} , Fe(II) , Co(III) , Pt(II) , and HgCl_2 . Although these systems add little directly to our understanding of the relationship between structure and function of the enzymes, nonetheless they represent new bioinorganic compounds and are of interest in themselves, or can add information on the coordinating capabilities, and reactivity in general, of the residues present in the active cavity.

Under the heading *zinc enzymes* there are several enzymes in which zinc is essential for the biological function, but is not present in the catalytic site. Among the most-studied enzymes, zinc has a structural role in superoxide dismutase, where the ligands are three histidines and one aspartate. In alcohol dehydrogenase there is a zinc ion that has a structural role, besides the catalytically active one. The former zinc has four cysteine ligands. Cysteine ligands are also present in zinc thioneins, which are zinc-storage proteins. The recently discovered class of genetic factors containing "zinc fingers" are zinc proteins in which the metal has an essentially structural role.²⁹ Such a role may consist of lowering the folding enthalpy of a protein to induce an active conformation or to stabilize a particular quaternary structure.

Zinc may also have a regulatory role; i.e., it does not participate in the various catalytic steps, but its presence increases the catalytic rate. This is a rather loose but common definition. Typically, zinc in the B site of alkaline phosphatase (Section V.B) has such a role, and the ligands are histidines, aspartates, and water molecules.

The enzymes in which zinc plays a structural or regulatory role will not be further discussed here, because they do not participate in the catalytic mechanisms; see the broader review articles.^{23,29,30} Rather, we will describe in some detail the enzyme carbonic anhydrase, in order to show how researchers have investigated such complicated systems as enzymes. We will discover as we look

at the details of the structures and mechanisms of enzymes that there are large differences between reactivities in solution and in enzymatic cavities. The fundamental properties underlying these differences are still not fully understood.

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