Synchrotron Radiation: New Window on Metalloprotein Structure

Many important enzymes are metal-containing proteins. Because the metals are intimately involved in the enzymes' catalytic activity, biochemists who are trying to find just how the enzymes work need as much detailed information as possible about the environment in which the metals are located.

They were provided with a new tool for probing these environments some 4 years ago when the Stanford Synchrotron Radiation Laboratory (SSRL) opened. The tool is high-intensity x-rays that can be used for absorption spectroscopic studies of metalloproteins. The absorption techniques are now being applied to refine structures derived in x-ray diffraction studies and also to study other metalloproteins whose structures are largely unknown because the proteins do not crystallize well enough for diffraction analysis.

More conventional sources of x-rays are not suitable for determining absorption spectra of metalloproteins because they do not provide x-rays of high enough intensity over the broad range of wavelengths needed. But the SPEAR electron-positron storage ring at Stanford [*Science* **190**, 1074 (1975)] provides a continuum of x-rays with energies up to a maximum of about 30 keV and with intensities as much as a million times greater than those of x-rays from conventional sources. With these x-rays investigators can tune in on the metals—ranging from potassium to the actinide metals—in proteins.

X-ray absorption spectroscopy enables the investigator to examine the immediate environment—within a radius of about 3 to 5 Å—of the metals. It cannot be used, as x-ray diffraction can, to determine the entire three-dimensional structure of the protein. But the spectroscopic techniques have other advantages. They can be applied in circumstances where diffraction methods do not work, such as on proteins that do not crystallize or on materials in solution. In addition, bond lengths can be determined with a high degree of accuracy, to less than 0.01 Å in the best circumstances. With this kind of information, investigators hope to clarify the role of metals in proteins.

Two good examples of metalloproteins in which the metals are intimately involved in function are rubredoxin and hemoglobin. Rubredoxin is an iron-containing protein of bacteria. It serves to shuttle electrons back and forth in certain oxidation-reduction reactions during the course of which the iron is alternately reduced and oxidized. Hemoglobin is, of course, the oxygen-carrying protein of red blood cells. The protein contains iron, which is coordinately bonded to four nitrogens in the porphyrin ring of the heme group. It is the iron that actually picks up and releases the oxygen.

X-ray diffraction studies indicated that certain properties of these proteins might be attributable to strain in the bonds between the iron atoms and some of their ligands. But recent x-ray spectroscopic results make this possibility unlikely. Two groups of investigators, one including Brian Kincaid, Peter Eisenberger, and Robert Shulman of Bell Laboratories (Murray Hill) and the other including Edward Stern, Dale Sayers, and Bruce Bunker of the University of Washington, used an absorption technique—extended x-ray absorption fine structure (EXAFS)—to study rubredoxin.

The x-ray absorption spectrum of a metalloprotein includes two distinct regions, each of which can be used to derive information about the absorbing metal and its ligands. They are the absorption edge region and the EXAFS region. The former consists of discrete absorption bands caused by the excitation of an electron from an inner electron shell of the metal to higher energy shells. These absorption bands may be superimposed on another sharply rising band, which is the result of the excitation of the electron out of the metal into the surroundings. From the shape and position of the absorption edge it is usually possible to derive information about the oxidation state of the metal and, in some cases, about the geometry of the metal and its ligands and the nature of the ligands.

On the high-energy side of the absorption edge is the EXAFS region. As the excited electron moves out from the metal atom it interacts with electrons of surrounding atoms and may be reflected back toward the donor metal. The reflected electron wave interferes with the outgoing electron wave, either constructively or destructively. The resulting perturbations convey information about the types, distances, and number of atoms bonded to the metal. In effect, says Keith Hodgson of Stanford University, the metal atom acts as both a source and detector of the reflected wave.

Using EXAFS, the two groups of investigators measured the lengths of the bonds connecting the iron of rubredoxin to its four sulfur ligands. Diffraction data initially seemed to show that while three of these bonds were about 2.3 Å long, the normal length for an iron-sulfur bond, the fourth at 2.05 Å was abnormally short. This bond would be strained and might be the source of the unusual oxidation-reduction properties of rubredoxin. The EXAFS data showed, however, that all four bonds were close to 2.3 Å long, a result that was confirmed by improved x-ray diffraction data. Thus, none of the bonds is unusually strained.

Despite the fact that hemoglobin is one of the most thoroughly studied of all proteins there are still unanswered questions about how the molecule functions. For example, the completely deoxygenated form of hemoglobin has a much lower affinity for oxygen than does hemoglobin that has bound one oxygen molecule. (Hemoglobin consists of four protein subunits; each of them has an iron-containing heme group and can bind a molecule of oxygen.)

Investigators have never adequately explained how binding of the first oxygen increases the affinity of hemoglobin for oxygen and thus facilitates additional binding. Since the hemes are not in contact with one another, there must be some mechanism for transmitting the change through the molecule.

One explanation for the effect was based on the pioneering x-ray diffraction studies of hemoglobin by Max Perutz of the MRC Laboratory of Molecular Biology in Cambridge, England. This work indicated that the iron of deoxygenated hemoglobin was displaced 0.75 Å out of the heme plane; binding of oxygen caused the movement of iron back into the plane. The theory was that the displaced iron was less accessible to oxygen, but once the iron was forced back into the heme plane by binding of the first oxygen molecule, the resulting large motion could be propagated throughout the protein to the other heme groups. Consequently their affinity for oxygen would be increased.

When the group at Bell used an EXAFS technique to measure directly the distances between the iron and the nitrogens of heme, however, they found that

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the iron-nitrogen bonds in deoxygenated hemoglobin were only 0.07 Å longer than in the oxygenated form. Thus, they calculated that the iron could be displaced out of the heme plane of deoxygenated hemoglobin by a maximum of 0.2 Å. The Bell workers suggest that the distortion produced by removal of the oxygen involves the entire heme group and not just the iron atom.

Ferritin is another iron-containing protein of biological significance. It stores the iron and then releases it as needed for the synthesis of hemoglobin and other proteins requiring the metal. Ferritin contains an iron phosphate core $[(FeOOH_8(FeO \cdot OPO_3H_2)]$ surrounded by the protein chain. Little was known for certain about the structure of the core until Stern and his colleagues applied EXAFS techniques to the problem.

Stern says that in order to get a complete structure from EXAFS data you have to be lucky—and they were lucky. The EXAFS data showed that the iron's nearest neighbors are six or seven oxygen atoms at a distance of 2 Å. The oxygens are apparently arranged around the iron in a distorted octahedron. The next nearest neighbors, which are 3.3 Å distant, are six or eight iron atoms.

Although EXAFS gives information about the number of ligands and the distances between them and the metal, the technique does not permit the determination of the angles formed by the bonds in question. The Washington workers, however, were able to determine a plausible structure for the iron core of ferritin by combining the EXAFS data with information about the stoichiometry and density of the core.

They were pleased to learn that they could eliminate all possible three-dimensional arrangements of the iron atoms because calculations showed that materials with such structures would be much denser than the iron core is known to be. Moreover, only one two-dimensional structure was consistent with all the data. This has the irons, which are in a hexagonal arrangement, forming a sheet sandwiched between two layers of oxygen atoms in their distorted octahedral array. Stern thinks that the phosphates are attached at the edges of the sheet. Since there must be two phosphates for every 18 iron atoms, this would give a sheet some 60 Å across, a figure consistent with the known diameter of the ferritin core, which is 70 Å.

A metalloprotein of great interest to biochemists is the enzyme nitrogenase. It catalyzes nitrogen fixation, that is, the reduction of atmospheric nitrogen to ammonia. One reason for wanting to know how the enzyme works is the hope that the knowledge may help man to duplicate the feat of fixing nitrogen in mild conditions and possibly lead to the development of an inexpensive method for making nitrogen fertilizer.

Nitrogenase is actually a complex of two proteins. The largest, which is called the molybdenum-iron protein, contains two molybdenum atoms and approximately 24 iron atoms. It has a molecular weight of about 220,000. Although investigators believe that the molybdenum is involved in the catalytic activity of nitrogenase, virtually nothing is known about its state in the enzyme or how it functions. Conventional chemical methods reveal little about the molybdenum of nitrogenase. Moreover, the molybdenum-iron protein cannot be studied by x-ray diffraction because it does not form crystals suitable for x-ray crystallography. Thus, it is a good candidate for analysis by x-ray absorption spectroscopy, which is now being carried out by Hodgson and his colleagues.

Since so little was known about the state of the molybdenum in the molybdenum-iron protein, the Stanford workers began by studying a series of model molybdenum compounds of known structure in order to determine the capabilities and limitations of the x-ray spectroscopic methods, including both absorption edge and EXAFS techniques. They found that they could determine the distances between the metal and its ligands with an accuracy of 0.03 Å or better. They could also distinguish the number and type of bound atoms with a reasonable degree of certainty. In particular, they could tell the difference between oxygen and sulfur, although not between oxygen and nitrogen.

Hodgson and his colleagues then applied the methods they had tested on the models to the molybdenum-iron protein isolated from the bacterium *Clostridium pasteurianum*. They concluded that sulfur atoms constitute the first shell of ligands bound to molybdenum. They could also detect an interaction between molybdenum and another metal, most likely iron. They thus suggest that the molybdenum is present in a cluster with sulfur and iron; the sulfur atoms form bridges between the two metals.

The investigators did not detect any oxygen doubly bonded to the molybdenum in the active enzyme, but they did find it in enzyme that had been air-oxidized. Formation of this bond is apparently involved in the rapid inactivation of nitrogenase by exposure to air.

According to Hodgson, the kind of structures they have proposed for molybdenum in the molybdenum-iron protein are unusual. None of the synthetic molybdenum compounds currently used as models for the enzyme have similar structures.

The Stanford group has recently obtained x-ray absorption spectra for the molybdenum-iron protein from the bacterium *Azotobacter vinelandii* and for another, smaller molybdenum-iron protein needed for nitrogenase activity. Hodgson says these spectra are very similar to those of the *C. pasteurianum* protein, a result suggesting that the structure of the molybdenum environment is the same in all three proteins.

The investigators who are using synchrotron radiation for x-ray absorption studies of metalloproteins are enthusiastic about the results they are obtaining and about the prospects for future research. In addition to the proteins mentioned here, they are studying, among others, the enzyme carbonic anhydrase, certain of the electron-transport proteins called cytochromes, and hemocyanin, the oxygen-carrying protein of arthropods and molluscs.

A major handicap at present, however, is lack of access to the appropriate facilities. In this country, only SPEAR can now produce x-ray beams of sufficiently high energy and intensity for most of the biological experiments. The x-rays produced by SPEAR are effectively by-products of the high-energy particle experiments being carried out by the physicists who run the machine. Researchers who wish to use the x-rays for absorption studies can do so only when the conditions they need happen to coincide with those produced by the high-energy experiments. In fact, the biologists sometimes describe themselves as "parasites" on the physicists.

This constraint should be eliminated, however, by the availability of new facilities that are being built at the University of Wisconsin and Brookhaven National Laboratory and by the expansion of SSRL. Moreover, some of these facilities will be dedicated solely to the production of synchrotron radiation. These three, in addition to a smaller facility at Cornell, will mean that 60—rather than the current half-dozen—experimental stations will be available for the x-ray work. This development in conjunction with certain technical advances that are now possible will permit the production of beams with higher energies and intensities—as much five to six times higher intensities—than are now possible. All in all, the future looks bright for the biological applications of synchrotron radiation.—JEAN L. MARX