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A MECHANISM ESSENTIAL TO LIFE

Scientists pursue consensus and closure on mechanism of binding of CO and O_2 to myoglobin and hemoglobin

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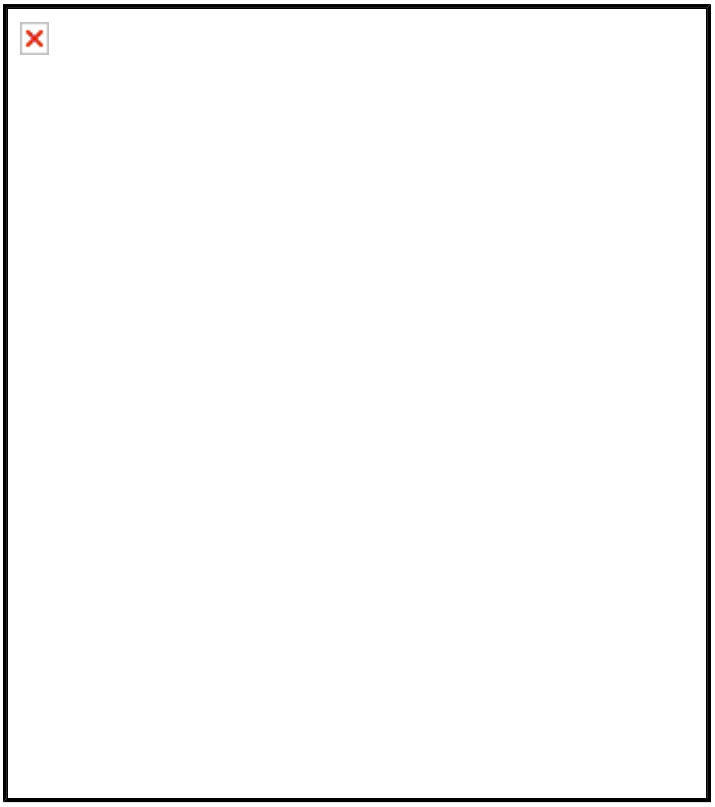
For several decades, researchers have been trying to understand how carbon monoxide and molecular oxygen bind to the heme proteins myoglobin and hemoglobin. With a new millennium about to dawn, they're still at it.

CO binds to free heme in solution with an affinity about 20,000 times that of O_2. But it binds to myoglobin and hemoglobin, respectively, with affinities only about 25 and 200 times those of O_2. This dramatic difference in relative affinities allows myoglobin and hemoglobin to function effectively as O_2 storage and transport proteins in the presence of CO--low levels of which are produced continuously in the body by normal heme breakdown and cell-signaling processes.



So far, the research community has not reached consensus on the mechanism by which myoglobin and hemoglobin discriminate against CO binding and/or favor O_2 binding. However, some researchers believe the mechanism is now evident and that consensus is only being blocked by opposition from a few stubborn holdouts.

Why bother arguing over the mechanism? Partly because "it's there," as mountaineer George Mallory once said of Mount Everest. But also because of the important roles the proteins play in carrying oxygen in blood (hemoglobin) and muscle (myoglobin). If CO's binding advantage (relative to O_2) were as big in heme proteins as in free heme, O_2 would be shut out of myoglobin and hemoglobin binding sites and organisms wouldn't be able to absorb O_2. The tendency of heme proteins to discriminate against CO binding is thus essential for life as we know it.

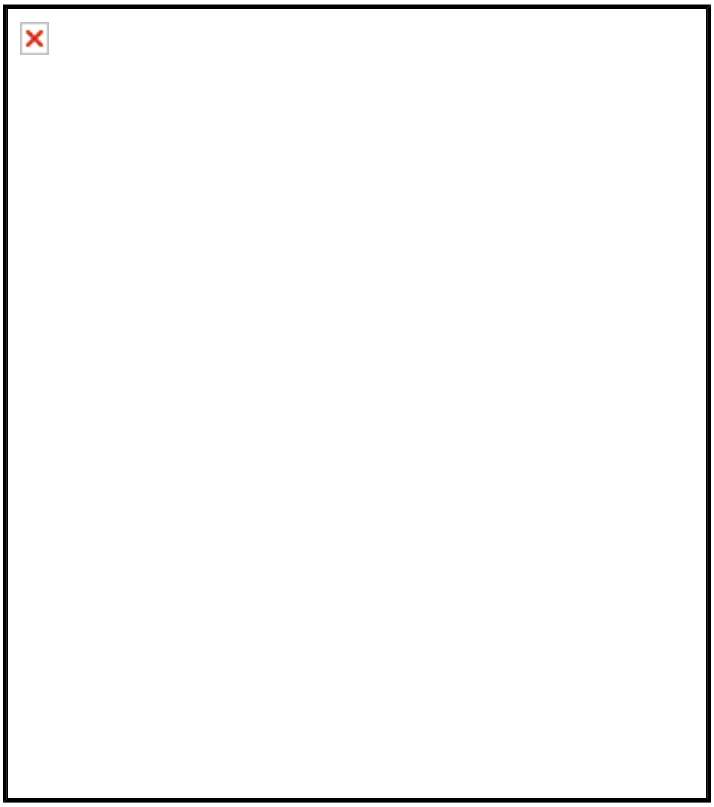
Determining how myoglobin and hemoglobin work is "crucial for understanding ligand discrimination by heme proteins in general and for engineering these proteins for various pharmaceutical purposes," says professor of biochemistry and cell biology John S. Olson of Rice University, Houston.

Steric destabilization of CO

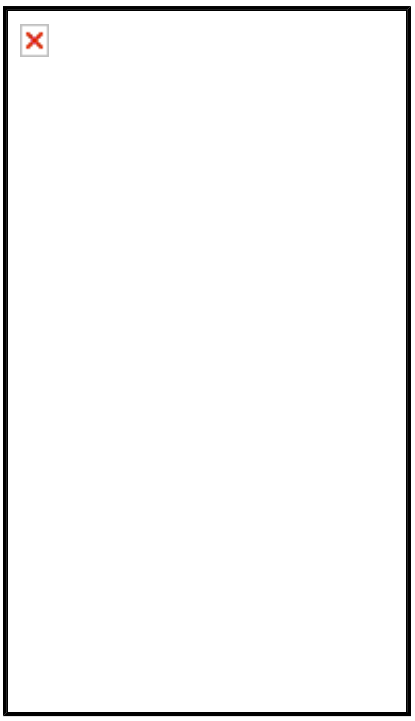
The dramatic difference in the relative affinities of CO and O_2 in free heme groups and in heme proteins was first noted by Stanford University chemistry professors James P. Collman and John I. Brauman and then-graduate students T. R. Halbert and Kenneth S. Suslick [*Proc. Natl. Acad. Sci. USA*, **73,** 3333 (1976)]. Since then, the most widely accepted explanation has been that amino acid residues in the heme proteins push CO around, destabilizing bound CO by steric hindrance.

It's this proposal that's at the heart of the current controversy. But at the time it was first devised, it was a very reasonable conclusion based on the experimental evidence available.

That evidence first emerged around 1970, when X-ray and neutron crystal structures of CO-bound myoglobin were obtained by several groups. They showed that, instead of sticking up straight from the heme plane, the CO ligand (FeCO group) was bent over by 40 to 60--that is, the angle between the Fe-C and C-O bonds was 120 to 140 instead of 180. That's a lot of distortion compared to the geometry one usually finds in CO-bound transition-metal complexes.



Based on this crystallographic evidence and studies of model porphyrin complexes, Collman and coworkers proposed in their 1976 *PNAS* paper that CO is distorted in heme proteins because the protein structure impinges on it sterically. They hypothesized that the distal histidine--a protein residue highly conserved in heme proteins and close to the bound ligand--forces CO into a bent orientation. O_2 is not affected by this bullying behavior on the part of the protein because it is always bent over in iron complexes anyway.



Study by Oldfield and coworkers of metalloporphyrins shows relatively small negative electrostatic potential (light blue) on the oxygen atom in CO and relatively large electrostatic potential (dark blue) on the nonbridging oxygen of O_2. The researchers believe that in heme proteins such a strong negative charge on O_2 favors hydrogen bonding between O_2 and the distal histidine, enhancing the binding affinity of the O_2 ligand.

"O_2 is always bent because of its diamagnetism," Collman explains. "The bound O_2 group is a superoxide ion. . . . The single unpaired electron spin couples with the unpaired electron on low-spin Fe, which requires a bent orientation."

Hence, O_2 and CO were both bound in a bent configuration in heme proteins--which was normal for O_2 but problematical for CO. The ratio of CO to O_2 binding affinities thus decreased in going from the ligands' complexes with free heme in solution to their complexes with heme proteins. The steric proposal seemed plausible, its validity became widely accepted, and it soon made its way into some college biochemistry textbooks.

FeCO: Bent or almost straight?

But questions have arisen about whether CO in carboxymyoglobin is really as bent out of shape as early crystallographic studies indicated. "Some of the early structures are just not that accurate," says chemistry and biophysics professor Eric Oldfield of the University of Illinois, Urbana-Champaign, "but these are the things that students read about."

Chemistry professor James A. Ibers of Northwestern University, Evanston, Ill., says: "The interpretation of severe bending of FeCO from the original X-ray and neutron carboxymyoglobin structures is something an inorganic chemist would say is complete nonsense. Physically, it is not

reasonable to bend a C-O bond by the 40 to 60 discussed in those original structure papers. There's no precedent for it in inorganic chemistry."

In a 1994 study, chemistry professor Thomas G. Spiro and coworkers (including Ibers) at Princeton University calculated that in the early X-ray and neutron structures of carboxymyoglobin the bend angle of FeCO had only been determined to a precision of 24 to 27.

"These errors were so large that you couldn't take seriously the conclusions of the structural studies about FeCO bending," Ibers says. "The protein structures didn't support the view that got into most of the textbooks. We and others have looked at a large number of model complexes, and in none of those have we seen distortion of FeCO of more than 6 or 7"--much less than the 40 to 60 bends seen in the early crystal structures.

Vibrational spectroscopy also now points to nearly straight FeCO units. In 1994, Spiro and coworkers found the vibrational spectra of carboxymyoglobin to be inconsistent with severe distortion. Also in 1994, physics professor Paul Champion and coworkers at Northeastern University, Boston, including postdoc Timothy Sage (now assistant professor of physics at Northeastern), published infrared crystallographic results on carboxymyoglobin indicating that the C-O bond lay fairly close to the heme normal (a line perpendicular to the heme plane).

"At the time, almost everyone accepted the tremendously distorted geometries reported in the X-ray structures," Sage says. "As a result, we faced considerable skepticism."

In 1995, a group led by Philip A. Anfinrud, now senior biomedical research scientist in the Laboratory of Chemical Physics at the National Institutes of Health, Bethesda, Md., performed a time-resolved IR dichroism study on carboxymyoglobin solutions that showed the FeCO group in carboxymyoglobin to be bent about 7. And in 1997, Sage and coworkers determined the C-O bond's displacement from the heme normal to be about 6.7.

IR studies such as those by the Champion, Sage, and Anfinrud groups measure the IR transition dipole of the CO bond. Spiro and Pawel M. Kozlowski (now assistant professor of chemistry at the University of Louisville) later showed computationally that the IR transition dipole is not necessarily colinear with the CO bond vector, as had been assumed. Nevertheless, they concluded that the deviation from colinearity is not large. So the IR studies' conclusion--that there is little geometric distortion of the CO ligand in heme proteins--remains valid.

Using density functional methods to analyze nuclear magnetic resonance, Mössbauer, electric-field gradient, and IR vibrational data, Oldfield and coworkers also have determined the FeCO bend angle to be about 7.

Recent crystallographic results likewise differ with findings from the early crystallographic studies on heme proteins. Hans D. Bartunik and coworkers at the Max Planck Research Unit for Structural Molecular Biology, Hamburg, Germany, obtained a high-resolution synchrotron structure of carboxymyoglobin showing an FeCO bend angle of about 7.4. A lower resolution carboxymyoglobin crystal structure obtained by Olson, professor of biochemistry and cell biology George N. Phillips Jr., and coworkers at Rice likewise indicates that the FeCO group is bent by about 7. And a low-temperature, high-resolution crystal structure of carboxymyoglobin obtained by biophysicists Joel R. Berendzen of Los Alamos National Laboratory and Ilme Schlichting of the Max Planck Institute for Molecular Physiology, Dortmund, Germany, and coworkers shows the FeCO bend angle to be about 9.

Minor quantitative discrepancies may thus remain, but "the controversy regarding the orientation of CO in carboxymyoglobin is over," Anfinrud says. CO does not bind at a severely bent orientation, as indicated in the earlier structures, and FeCO distortion is evidently too small to be the primary factor influencing CO binding affinity.

Inhomogeneity of the heme protein crystals studied could be one reason for problems with the early structures. Crystallographer Gregory A. Petsko of the department of chemistry at Brandeis University, Waltham, Mass., believes the early X-ray and neutron studies "were almost certainly looking at mixtures of species." However, "they represented the best that could be done at the time," he says. "The times have changed and so has our ability to look at fine details of structure and to trap metastable species. It's reasonable and appropriate that these structures should get revisited with modern methods."

What Collman and coworkers did "was to model a system provided by crystallographers," chemistry professor Steven G. Boxer of Stanford University says. "They did so beautifully. . . . The problem is that the model isn't a model for the real protein, because the early X-ray data were not accurate enough."

Hydrogen bonding and electrostatics

But if steric destabilization isn't causing heme proteins to discriminate against CO binding, what is?

The now-prevailing alternative hypothesis is that hydrogen bonding and electrostatic field interactions in heme proteins account for the ligand discrimination. Essentially, the proposal is that O_2 is preferentially stabilized by hydrogen bonding and electrostatic forces, and that it is thus O_2 stabilization instead of CO hindrance that accounts for the proteins' discrimination (relatively speaking) against CO binding.

In 1964, the late chemistry professor Linus Pauling first proposed that bound O_2 was preferentially stabilized by hydrogen bonding to the distal histidine. And beginning in the early 1980s, several research groups--including those of Suslick, now chemistry professor at the University of Illinois, Urbana-Champaign; Olson; the late Teddy G. Traylor of the department of chemistry at the University of California, San Diego; and the late Michel Momenteau of the research division of the Curie Institute, Orsay, France--proposed that electrostatic polarity effects might be more important than steric hindrance in the mechanism of ligand binding to heme proteins.

Key results that led to the development of a quantitative electrostatic hypothesis for ligand discrimination came from site-directed mutagenesis experiments by several groups, including those of Olson; Boxer; Kiyoshi Nagai of the structural studies division at MRC Laboratory of Molecular Biology, Cambridge, England; biochemistry professor Stephen G. Sligar at the University of Illinois, Urbana-Champaign; Anthony J. Wilkinson at the department of chemistry of the University of York, England; and physiology and biophysics professor Masao Ikeda-Saito at Case Western Reserve University School of Medicine, Cleveland.

"When the distal histidine of myoglobin is replaced by nonpolar residues, oxygen affinity decreases about 100-fold, whereas CO affinity only increases on average about fivefold," Olson explains. "Thus, the distal histidine is not hindering CO binding very much, but rather stabilizing bound oxygen" by hydrogen bonding and electrostatic forces.

Recently, Phillips, Olson, and coworkers used a combination of theoretical calculations, IR spectroscopy, X-ray crystallography, and further mutagenesis studies to put the electrostatic stabilization hypothesis on a firm footing. And Boxer and coworkers have used vibrational Stark spectroscopy to quantify the connection between electrostatic parameters (such as mutation-induced changes in electric fields) and the mechanism of ligand discrimination in heme proteins.

Density functional theory calculations by Spiro's group and others have suggested that steric forces in myoglobin can account for no more than one-third of the protein's discrimination against CO and in favor of O_2. "The rest must be electrostatic," he says. "This is consistent with Olson's binding studies on myoglobin mutants."

In addition, Oldfield and coworkers recently showed that the nonbridging oxygen atom (the one not bound to Fe) in an oxymyoglobin model compound has a significantly larger negative charge buildup than the FeCO oxygen atom in a carboxymyoglobin model. Oldfield notes that such an enhanced negative charge favors stabilization of O_2 binding in hemoglobin and myoglobin by hydrogen bonding of the ligand to the distal histidine.

Although there is a growing consensus that stabilization of O_2 binding is more important than destabilization of CO binding, steric destabilization may still play a significant role. "We have been looking in the wrong place for the steric contributions," Sage says. "Instead of the protein ganging up to distort the poor helpless CO, the main steric cost of CO binding to myoglobin must be the distortion of the surrounding protein to accommodate a nearly upright CO. Until the energy involved has been evaluated quantitatively, it is too soon to close the door on steric control of CO versus O_2 discrimination."

Collman insists that the case for hydrogen bonding and electrostatic field interactions as sources of discrimination against CO binding has not been nailed down. "Everyone agrees that bound O_2 has excess negative charge on the terminal oxygen atom, but the role of hydrogen bonding in the heme proteins is complex, and work on hydrogen bonding with models is not yet clear. Thermodynamic and structural work on several model iron porphyrins unambiguously demonstrates that steric interactions can dramatically lower the affinity of the models for CO, while leaving the O_2 affinity unchanged. To dismiss steric interactions in the hemes because the actual distortion of the FeCO group is small is to put one's head in the sand. Something really does lower the CO affinity in hemoproteins."

Closure?

Oldfield notes that "it might be nice to bring some closure to at least some of these decades-old structural questions" on ligand discrimination in heme proteins. "The combination of NMR, Mössbauer, IR, and quantum chemistry together gives only one conclusion"--that the steric destabilization hypothesis is dead and the hydrogen bonding and electrostatic hypothesis should now displace it in the biochemistry textbooks. "That's the bottom line," he says.

Olson agrees that "the ligand-discrimination problem really is solved, the paradigm has been rewritten, and the biochemistry textbooks need to be revised to present the latest facts and interpretations."

However, Collman reiterates that he doesn't believe "that this complex problem is at closure. There

is no general agreement about the various aspects of this Gordian knot."

According to Ibers, "The early neutron and X-ray structures and the steric hypothesis called attention to an interesting and important aspect of heme chemistry. As a result, sophisticated modeling and spectroscopy ensued. All this is good, and the net result is that we're all smarter now. If only the biochemistry textbooks would change their story!"

The critical importance of the steric destabilization hypothesis, Suslick says, "was the realization that there was discrimination between CO and O_2 binding in heme proteins. The mechanism by which this occurs almost certainly involves multiple components: local polarity, hydrogen bonding, and steric demands of the binding site. The quantitative contributions of each of these remains to be fully resolved."

There is no doubt, Suslick says, "that heme proteins cannot bind CO in a linear, nontilted fashion without something moving out of the way. I think all crystallographers would agree on this. The issue that has made all the fuss was the assumption that it was the FeCO unit that gave way and distorted. This has now been revisited, and it may well be that it is the peptide structure that adopts a slightly different conformation. Either way you get a steric component that will favor O_2 binding relative to CO binding. Any controversy that's left in this area is just the seven blind men describing the elephant."

Petsko agrees that "this seems like one of those cases where everybody's right and that the difference is everybody is looking at a slightly different sample under slightly different conditions with different techniques. It was like that in immunology. Remember about 20 years ago when everybody had different ideas on how the immune system generates diversity? There were three main speculations. Well guess what? All three turned out to be right." For the mechanism of ligand binding in heme proteins, he says, "I think probably everybody's right and the trouble is they're not exactly comparing the same things. It has that kind of air about it to me."

But several other researchers do not believe everybody is right. Anfinrud, referring to recent crystallographic results on the structure of heme proteins, says: "We have come full circle. The classic tale of CO discrimination in myoglobin, taught in many biochemistry textbooks, is ultimately undone by the same experimental method that generated that paradigm. There is something quite compelling about the beautiful glassy spheres that depict the atomic positions in 3-D protein structures, and it is reassuring that the carboxymyoglobin crystal structure is now consistent with a host of other experimental and theoretical findings. Perhaps the carboxymyoglobin story will provide an alternative tale of how one set of glassy spheres, after substantial prodding by a host of biophysicists and physical chemists, morphed into another."

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