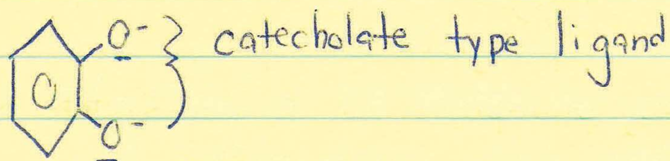
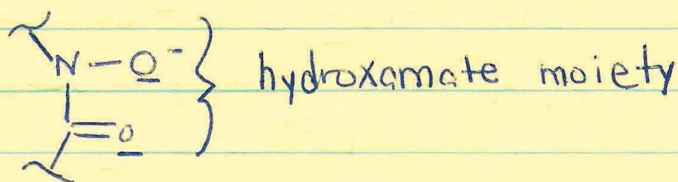
Assignment 3 Answer Key

1) Siderophores are metal chelators that are secreted by organisms such as bacteria and fungi to mobilize iron as Fe^{3+} and transport the metal into them.

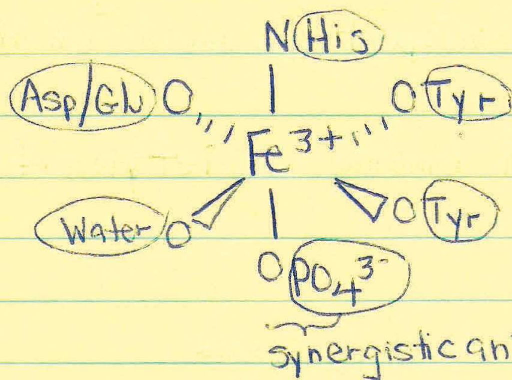
The general metal binding moieties of these small molecules are





In Gram-positive bacteria, in contrast to Gram-negative bacteria (see the attached figure), there is a single membrane surrounded by peptidoglycan. These bacteria have a lipoprotein on the cell's outer surface that binds Fe^{3+}-bound siderophores. The Fe^{3+}-siderophore is then imported by a transmembrane protein (siderophore-permeases) and ATPase.

2. The bacterial ferric binding proteins have a metal binding site that is comparable to that of the transferrins. It is illustrated below:



In these proteins, phosphate as a monodentate ligand serves as a synergistic anion rather than carbonate. The interesting thing is that monodentate or even bidentate phosphate is unlikely to substitute as a synergistic anion for Fe^{3+}-binding in transferrins.

As bacteria can be human pathogens, it is believed that bacterial ferric binding proteins serve the function of acquiring Fe^{3+} from human serum transferrin. These proteins are found in Gram-negative bacteria in the periplasmic space. They are typically half the size of bilobal transferrin, with a single Fe^{3+} metal binding site, which has a comparable affinity for Fe^{3+} as does human serum transferrin. Unlike other periplasmic-binding proteins that coordinate Fe^{3+} as a chelate, FBP coordinate Fe^{3+} as an ion.

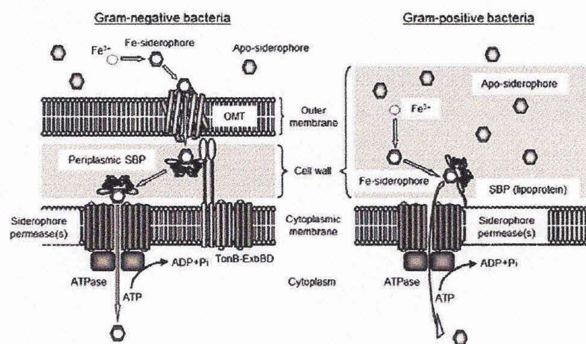


Fig. 1. Siderophore uptake machineries in Gram-negative bacteria (*Left*) and Gram-positive bacteria (*Right*). Gram-negative bacteria possess an OMT of Fe-siderophores. After an OMT recognizes a siderophore, the Fe-siderophore is transferred to a periplasmic SBP using the TonB-ExbBD system (5). The Fe-siderophore bound to the SBP is then imported using the appropriate siderophore-permeases and ATPase. In Gram-positive bacteria, a lipoprotein SBP anchored to the membrane binds a siderophore, and the Fe-siderophore is imported using its siderophore-permeases and ATPase.

as to enable iron exchange (15), although the nonfacilitated iron exchange rate between Fe- and apo-hydroxamate siderophores is extremely slow, with a half-life of nearly 10 d (16). The newly formed Fe-siderophore continues into the cell and the former Fe-siderophore remains in the OMT as an apo-siderophore, ready to participate in the next siderophore-shuttle (14).

The siderophore-shuttle in Gram-negative bacteria depends on the ability of OMTs to bind apo-siderophores. For the Gram-positive siderophore shuttle mechanism, a lipoprotein SBP binds an apo-siderophore (Fig. 2). Then a Fe-siderophore interacts with the SBP near the apo-siderophore. The increased local concentration complex facilitates iron exchange to the apo-siderophore, and the new Fe-siderophore is passed through the permeases to the cytoplasm (Fig. 2*C*). The alternative uptake mechanism, when an apo-siderophore is initially bound to the SBP, we will call the "displacement mechanism" (Fig. 2*D*). In this mechanism, the Fe-siderophore displaces the apo-siderophore from the SBP. No iron exchange takes place, and the original Fe-siderophore passes through the permeases to the cytoplasm. Iron exchange is the distinguishing feature of the two mechanisms.

B. cereus ATCC 14579 uses a lipoprotein SBP called YxeB to bind and import FO (ferrioxamine B) and Fch (10). These two siderophores deliver iron through YxeB, even though *B. cereus* does not produce the corresponding apo-siderophores DFO (desferrioxamine B) and DFch (17). We report that YxeB uses a Gram-positive siderophore-shuttle mechanism to transport Fe-siderophores when apo-siderophore is present.

Results

YxeB Binds DFO, FO, DFch, and Fch. Previously, Zawadzka et al. demonstrated that YxeB (BC_0383) binds Fe-siderophores, FO and Fch (10). However, it was unknown if the protein also binds apo-siderophores, DFO or DFch (Fig. S1*A*). To begin our study of YxeB, the *yxeB* gene in *B. cereus* ATCC 14579 was sequenced. Sequencing revealed two different nucleotides in the gene compared with the sequence in the National Center for Biotechnology Information (NCBI). One nucleotide, G_{555}, in the database (the number is with respect to the first nucleotide of the *yxeB* translational start codon) is incorrect, and the correct nucleotide is A_{555}. The other nucleotide has two variations, $TT_{425}A$ and $TC_{425}A$, in the laboratory stock. The *yxeB* genes with $TT_{425}A$ and $TC_{425}A$ encode YxeB-L142 (residue 142 is Leu) and YxeB-S142 (residue 142 is Ser), respectively. Both YxeB-L142 and YxeB-S142 were used in the following fluorescence-quenching assays to measure the binding affinity for several substrates.

The quenching assays of YxeB-L142-6×His show that the protein fluorescence was quenched by FO and Fch (Fig. S1 *B* and *C*). The data were fit to a one-to-one binding model using Hyperquad (18) to determine K_ds. The K_ds for FO and Fch were 38.8 nM and 43.0 nM, respectively (Table 1). Significantly, the protein fluorescence increased upon addition of DFO or DFch, the same as previously reported by Zawadzka et al. for YxeB-V5 (epitope tag)-6×His (10). Thus, it is possible that the increasing fluorescence of the YxeB-L142 protein is caused by substrate binding. To confirm this theory, nano–ESI-MS (electrospray ionization-mass spectrometry) analysis of the protein and DFch or Fch complexes was performed. The data show that the protein formed complexes with DFch and Fch (Fig. S2 and Table S1). Additionally, the YxeB-L142 protein mixed with DFO or FO was purified and then analyzed by reverse-phase (RP)-HPLC showing that the protein had bound DFO and FO (Fig. S3 *B* and *F*). Thus, it is clear that the increasing fluorescence of the protein is because of siderophore binding.

A fluorescence-quenching assay of the YxeB-S142 protein was also performed. The fluorescence was quenched by DFO, FO, DFch, and Fch (Fig. S1 *D* and *E*) and the calculated K_ds for the substrates by Hyperquad (18) were very similar (Table 1). Thus, YxeB-S142 has similar affinity for both the apo and ferric forms of siderophores.

YxeB Is the Sole FO/Fch-Binding Protein. *B. cereus* ATCC 14579 produces only PB and BB (bacillibactin), yet it possesses at least 10 genes encoding siderophore binding proteins (17) (*B. cereus* ATCC 14579 gene annotation in the NCBI genome database). It has been demonstrated that the gene products YxeB, YfiY, FeuA, and FpuA/FatB are a DFO/Fch, schizokinen, Ent/BB, and PB (FpuA and FatB)-binding proteins, respectively (10). FctC was recently identified as a triferric tricitrate-binding protein (19). The FO/Fch-binding ability of the other less characterized siderophore-binding proteins of *B. cereus*, BC_2208, BC_4363, BC_4416, and BC_5380, was assessed, and none of these proteins bind FO or Fch (Fig. S4). Thus, YxeB is the only DFO/Fch-binding protein in vitro.

To confirm that YxeB is the only DFO/Fch-binding protein in vivo, the *yxeB* markerless mutant was constructed (*Materials and Methods*). Because *yxeB* and the downstream genes, *BC_0382* and *BC_0381*, make an operon, only *yxeB* is disrupted in the constructed strain, TC111 (*yxeB*$^-$), while preserving the downstream genes. Fig. S5 *A* and *B* show the growth assay of TC111, TC129 (YxeB-L142), and TC128 (YxeB-S142) strains. This assay uses iron-limited minimum medium, but DFO can chelate Fe (III) from the medium even though the iron concentration is very low. The growth of TC129 and TC128 with DFO was better than the growth without DFO, indicating that both the strains can import and use FO. On the other hand, the growth of TC111 with DFO was not better than the growth without DFO, showing that the TC111 strain cannot use FO. This result also shows that PB and BB produced by *B. cereus* during the experiment do not affect growth in these conditions.

To further assess whether TC111 can use FO and Fch or not, a disc-diffusion assay was performed. In this experiment the cells grow around a disc containing FO or Fch if the substrates can be used. As shown in Fig. S5*C*, the wild-type strains, TC129 and TC128, grew in halos around the discs containing FO and Fch. However, TC111 did not grow around the discs with FO and Fch, although the strain grew around a disc containing BB (the positive control substrate), indicating that it cannot use FO and Fch. Therefore, the in vivo growth assay and disc-diffusion assay strongly suggest that *yxeB* is the sole FO/Fch-binding protein. The other SBPs including YfiY, FeuA, FpuA, FatB, and FctC are not associated with FO/Fch uptake. Moreover, Fe(III) coordinated with FO is not transferred to BB and PB produced by *B. cereus* under the iron-limited condition because the *yxeB* mutant, TC111, did not grow well.

Cr-DFO Is a FO Analog. Because Cr(III) is kinetically inert and will not exchange from one siderophore to another on the experimental

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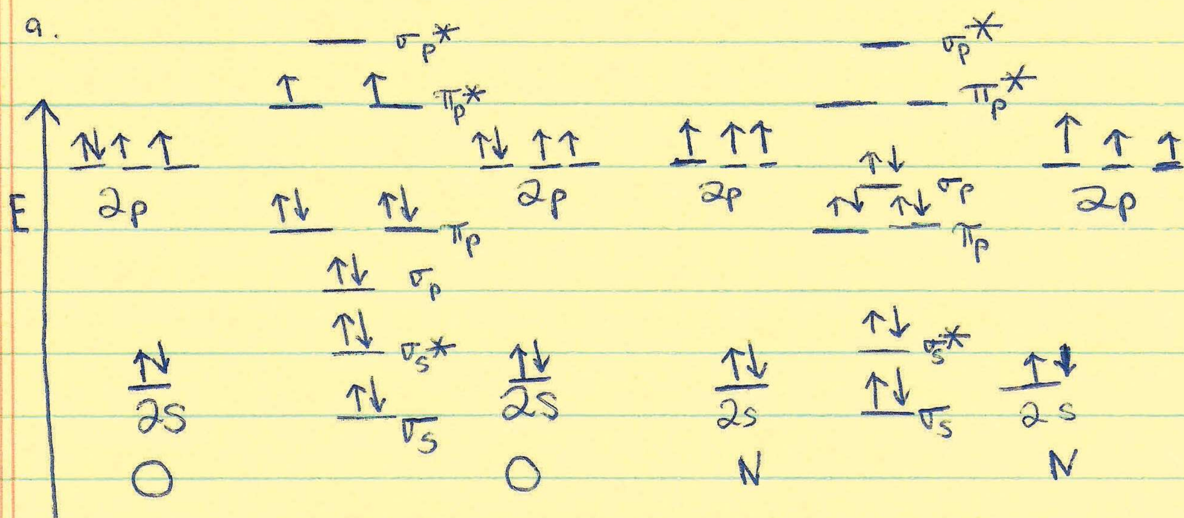
3. a.



would not be comparable in energy.

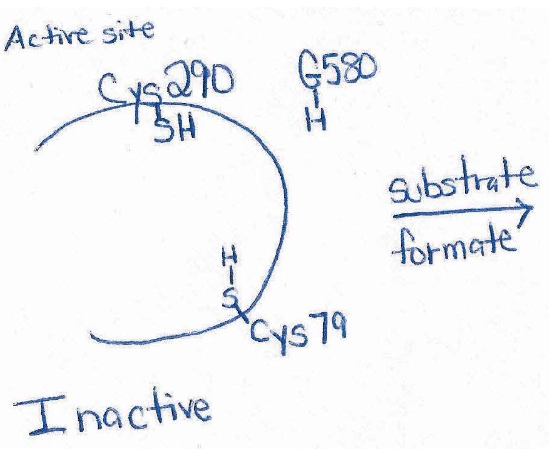
$B.O. = \frac{1}{2}[8-4] = 2$ $B.O. = \frac{1}{2}[8-2] = 3$

b. If you pair the π_p^* electrons in the M.O. diagram for O_2 then you would have no unpaired e^-. This requires energy to enable the spin flip and overcome coulombic repulsion.

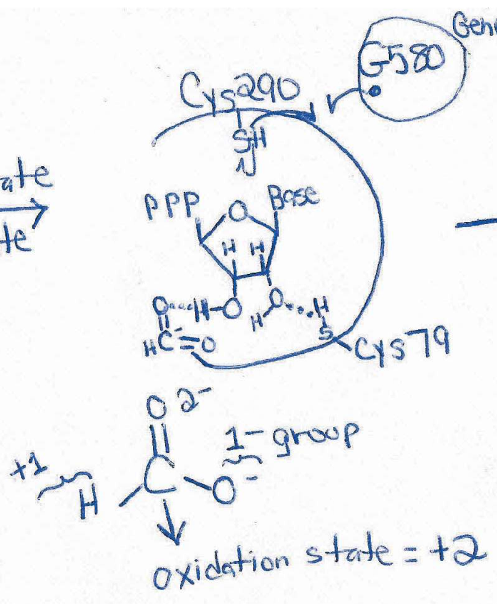
c. Imagine O_2^{4-} ⇒ You would ~~complete~~ fill the π_p^* and σ_s^* orbitals of O_2.

$B.O. = \frac{1}{2}[8-8] = \underline{0}$

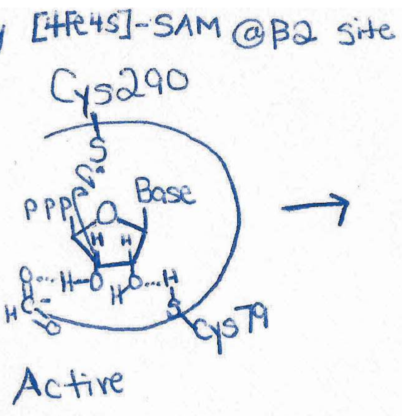
α2 Active site

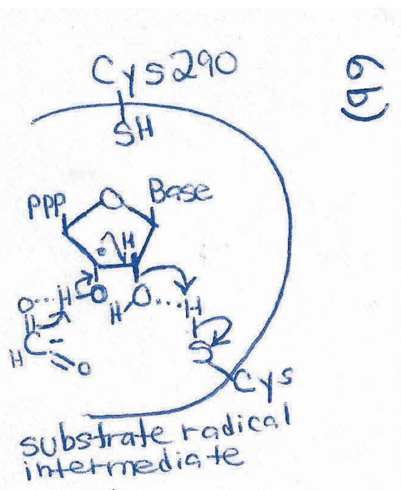


substrate
formate

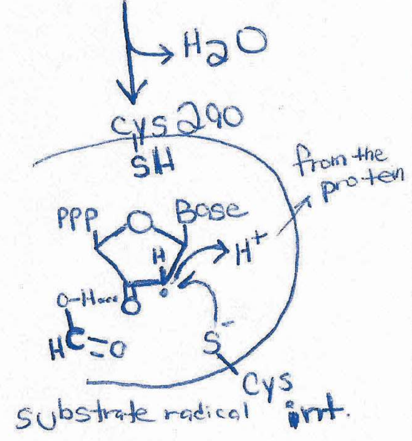


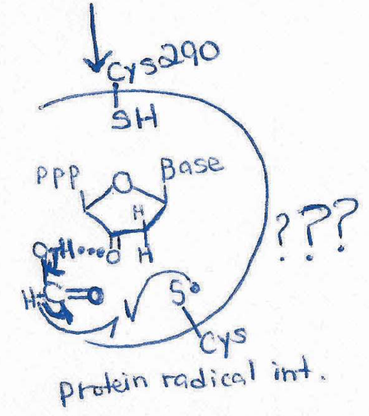
Generated by [4Fe4S]-SAM @B2 site



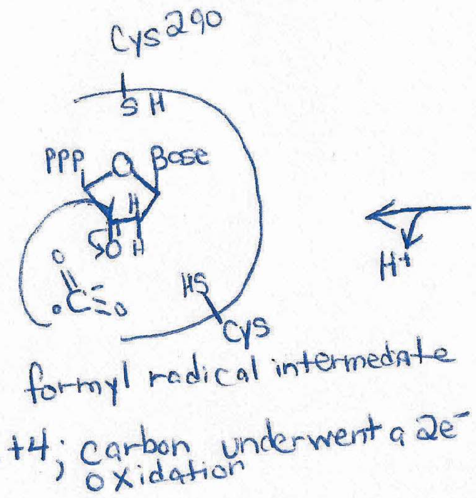


→ H_2O



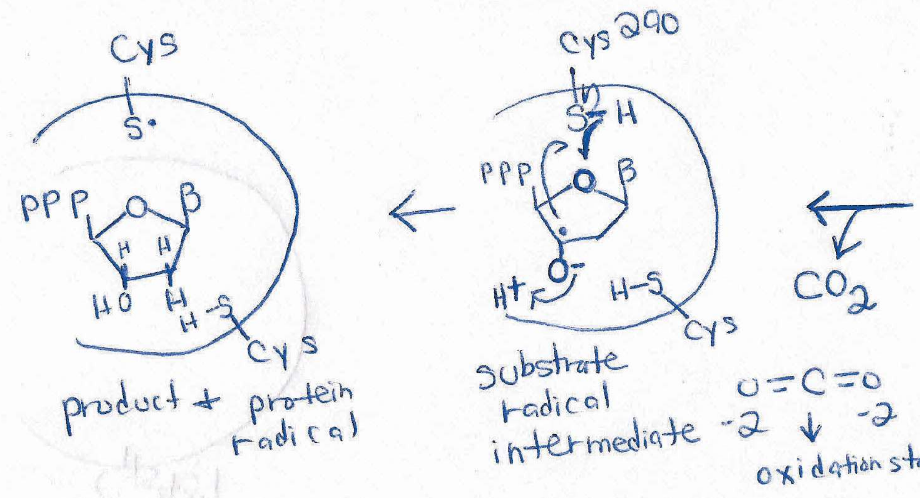


H+



CO_2

O=C=O
-2 ↓ -2
oxidation state = +4; carbon underwent a 2e⁻ oxidation



4.

5 a. Ga^{3+} is similar to Fe^{3+} in terms of dominance of its charge in an oxidizing environment, ionic radius, and very importantly, coordination preference. It is believed that Ga^{3+} could interfere with important Fe^{3+} pathways.

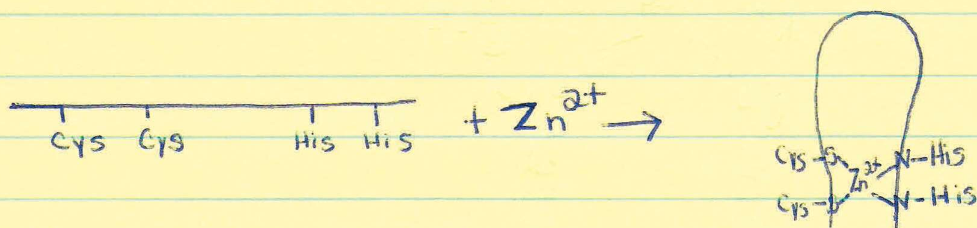
b. The electron configuration of Fe^{3+} + Ga^{3+} can greatly inform on the redox differences of these two ions.

There is an extremely high stability for having a fill n=3 shell and would be difficult to add or remove an electron.

c. If the R2 site of the class I RNR loses its two iron ions, the tyrosyl radical will quickly convert to tyrosine. Ther Ga^{3+} ions would then be able to bind to the diiron site but because of the redox inertness of Ga^{3+}, the tyrosyl radical would never be generated and

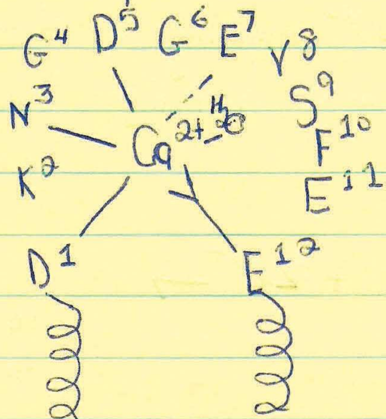
and therefore the active site thiyl would not be generated.

6. Zn finger domains are protein domains that contain a Zn finger structural motif or CCHH motif, which stands for 2 cysteines and 2 histidine amino acid ligands. These ligands are not present in a sequential order along the peptide sequence but at ~~a~~ two distinct parts of a strand. Zn^{2+} binding to these amino acids results in the formation of a finger loop structure.



The coordination geometry is typically distorted tetrahedral. Zn finger domains, like other structural Zn domains, play a significant role as interfaces for macromolecular interactions. They serve as interfaces for protein-nucleic acid interactions. The classical Zn finger domain is composed of two antiparallel β strands followed by an α helix, which when Zn^{2+} bound are placed in close proximity to one another creating a hydrophobic core. In these domains, the two cysteines are found in a β hairpin turn and the two histidines in the α helix. Three residues in the helix (not Zn^{2+} bound) interact with bases within the major groove of the duplex. Other residues form contacts with the DNA backbone.

7. To discuss Ca^{2+} binding to calmodulin and the physiological importance of this interaction, it is important to briefly discuss the Ca^{2+}-binding motif, the "EF-hand." The EF-hand Ca^{2+}-binding loop consists of 12 amino acid residues that are connected on both sides by an α-helix.



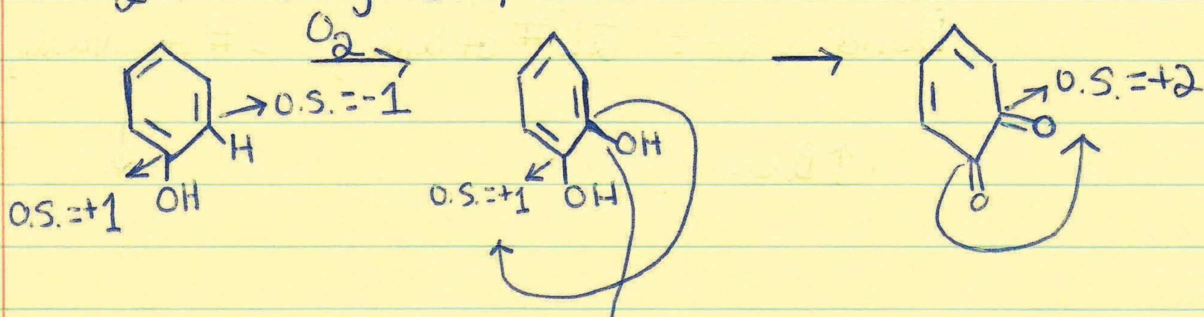
- D1, G6, and E12 are highly conserved amino acids in the motif.
- The side chains of residues 1, 3, 5, + 12 serve as ligands. The side chain of res. 12 serves as a bidentate ligand
- The backbone carbonyl of residue 7 and either the side chain of residue 9 or H_2O also serve as ligands. Therefore the coordination number for Ca^{2+} is 7, which yields a distorted octahedron or pentagonal bipyramid geometry.

The functional unit in proteins is a pair of EF-hands, which are stabilized by helical contacts between

the EF hands ("intermolecular" contacts). I say "intermolecular" because it is the helices from the different EF hands that interact.

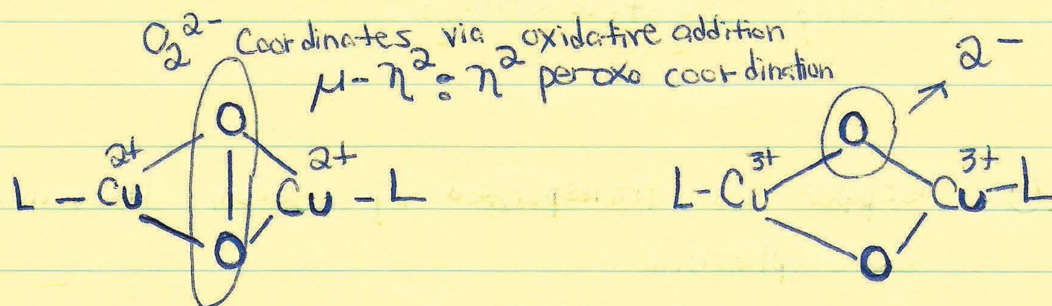
The EF hand Ca^{2+}-binding loop is employed by the protein calmodulin for bioactivity. This protein consists of two EF-hand pairs that in the absence of Ca^{2+} are Mg^{2+} bound for a total of 4 Mg^{2+}. The Mg^{2+} ions must dissociate for the 4 Ca^{2+} ions to bind. Ca^{2+} binding to calmodulin, unlike Mg^{2+} binding, results in a major conformational change that facilitates binding of other proteins to calmodulin. X-ray structures reveal that the two EF-hand pair domains could move relatively easy along one another so that they could accomodate interactions with many sequences of other proteins. This is important because calmodulin is a modulator protein. The Ca^{2+}-induced conformational change alters the orientation of the two helices within an EF hand from roughly antiparallel to more perpendicular and in the process, expose hydrophobic interactive surfaces. These surfaces can serve as recognition sequences for different target proteins. Some of these target proteins are enzymes. These enzymes only become activated when bound to the Ca^{2+}-bound form of calmodulin.

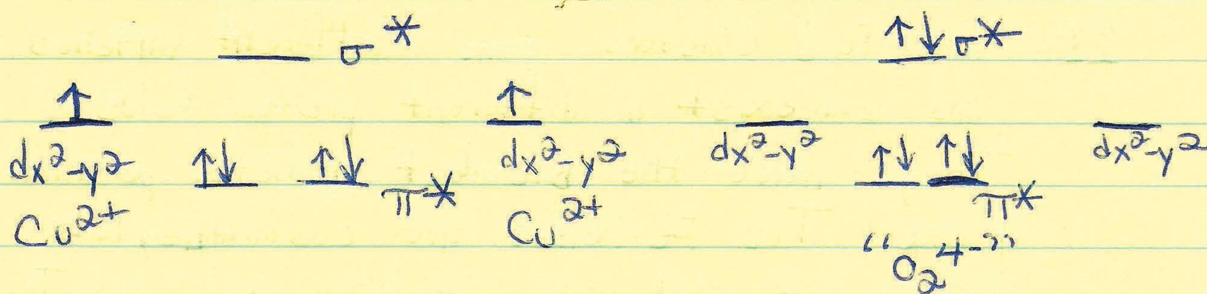
8. a. O_2 activating enzyme



- Addition of one oxygen into the C-H bond. Hence, a mono oxygenase.
- The other oxygen becomes a water molecule.
- The O atoms from O_2 are reduced.

b.



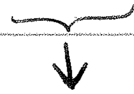


B. O. = 0

c. The preserve the O-O bond in the peroxo ligand, L has to be electron withdrawing because removal of e⁻ density from Cu^{2+} would then remore e⁻ density from the peroxo, effectively removing ~~this~~ some density from

an antibonding molecular orbital and strengthening the bond.

Bond order = $\frac{1}{2}$ [# of bonding e^-s − # of antibonding e^-s]



↑ B.O.

9. Effective O_2 if Fe^{2+} oxidation state is made stable by the protein environment. Poor O_2 carrier if Fe oxidation state is Fe^{3+} or can fluctuate between Fe^{2+}/Fe^{3+} due to coordination of an axial ligand with partial or fully negative charge.