Iron–Sulfur Proteins with Nonredox Functions

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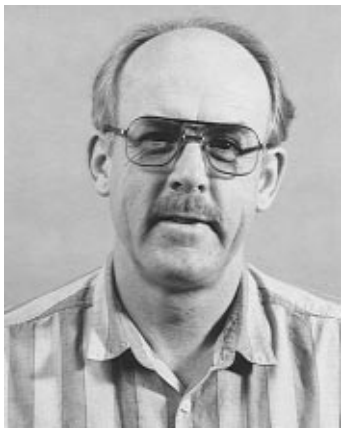

Dennis H. Flint was born and raised on a farm in Utah and after attending Utah State University for one year he served a two year mission in Manitoba for the Mormon Church. He graduated from Brigham Young University with a B.S. in Chemistry and from the University of California, Davis, with a Ph.D in Biochemistry. He received postdoctoral training in Joseph Varner's laboratory in the Plant Research Laboratory at Michigan State University. He worked at Shell Development Company's Biological Sciences Research Center in Modesto, CA, for 12 years and then joined the Central Research and Development Department of the DuPont Company. His research interests include the application of the principles of chemistry, biochemistry, and genetics for the betterment of farming. Other interests include helping disadvantaged youth make the transition to productive adults.

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I. Introduction

One of the joys of participating in biochemical research is being able to observe first hand the unexpected paradigm shifts that take place in science as surprising new findings are made. Two recent examples from the field of bioinorganic chemistry include the discovery of the biological role of nitric oxide and the structure of the FeMoCo cluster in the enzyme nitrogenase. A third example is the subject of this review article, the expanding list of nonredox functions performed by protein bound Fe-S cluster prosthetic groups.

Concomitant with their discovery over three decades ago, protein-bound Fe-S clusters were found to participate in electron transport.[1−4] In the ensuing years, several types of Fe-S clusters were discovered including [2Fe-2S] clusters, cubane and linear [3Fe-

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4S] clusters, cubane [4Fe-4S] clusters, and the magnificent Fe-S clusters found in nitrogenase. Rubredoxin, a protein with one Fe ligated by four cysteinyl sulfhydryl groups, is often considered a member of the class of Fe-S proteins even though it lacks acid-labile sulfide. Several members of the Fe-S cluster-containing class of proteins have been extensively studied and excellent reviews of earlier work in this field have been written.[5−8]

For several years the only known function for Fe-S clusters was the one discovered first, a participant in single electron-transfer reactions. These reactions are essential to many of the fundamental processes of life including photosynthesis, respiration, and nitrogen fixation. More recently, the list of functions began to expand and now includes in addition to electron-transfer reactions: (1) catalysis (where they act as Lewis acids in dehydration reactions); (2) stabilization of protein structure; (3) regulation of metabolic pathways; (4) biological sensor of iron, O_2, and O_2^-, and (5) a possible function in the formation of protein-bound radicals.[9,10] Proteins that perform functions 1 through 4 are the subject of this review.

A dominant theme will be how Fe-S clusters perform these functions. A minor but potentially seminal theme is why they have been chosen to perform these functions. The question why confronts us forcefully here because in several cases in which nature has chosen to use Fe-S clusters in nonredox functions, it has also devised a clusterless means of fulfilling the same functions. Why use Fe-S clusters if you can make do without them?

There is a line of reasoning that Fe-S clusters are evolutionary relics left over from the days when the levels of oxygen in the atmosphere were much lower than today.[11−13] It follows from that line of reasoning that some biological functions performed anciently by Fe-S clusters may have been abandoned during evolution and that some of the functions currently assumed by Fe-S clusters will eventually be replaced by more efficient metal-free systems. However, evidence will be presented in this review that suggests some Fe-S clusters perform multiple functions. These Fe-S clusters appear to be not so much evolutionary relics as efficient devices for which it would be difficult if not impossible to find a replacement. Maybe a multiple function modus operandi is more widespread than presently appreciated. If so, the seminality alluded to above will be realized.

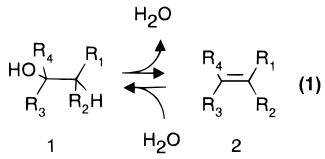
We would like to remind readers of the problems organisms face in dealing with iron. Although iron is the fourth most abundant element in the crust of the earth,[14] paradoxically one of the most significant problems organisms face is its acquisition. This is due to the insolubility of iron, the amount of uncomplexed ferric ion in oxygenated aqueous solutions near neutral pH being on the order of 10^{-18} M.[14,15] The concentration of iron in seawater where it exists primarily on suspended solids can range as low as picomolar (10^{-12} M) levels.[16] This is as much as a million times lower than the iron concentration commonly found in organisms.[14] Often iron is the nutrient that limits biomass production in ocean waters.[16−18] The elaborate lengths organisms go to acquire iron has only recently been appreciated.[19]

Iron problems do not end with its acquisition. Once it is inside a cell, free iron can be severely toxic due to its ability to participate in Fenton and other redox chemistry.[20−23] For these reasons the regulation of iron acquisition, storage, and use is elaborate. It seems that it would be advantageous for organisms to use iron only in functions where its unique properties were essential.

It has been fascinating to find in the last few years that in at least one case a protein with a Fe-S cluster participates in iron regulation.

II. Enzymes of the Hydro-lyase Family That (1) Contain a Fe-S Cluster That May Perform Catalytic and Regulatory Functions or (2) Require Iron for Activity

Enzymes in the hydro-lyase class [EC 4.2.1.*x*] catalyze the generalized reaction 1 in which the elements of water are removed across a carbon−carbon bond thereby converting an alcohol **1** into a compound with a vinyl group **2**. Enzymes in this class can be divided into at least two and perhaps three subclasses: (1) the hydro-lyases that do not contain or use metals in catalysis; (2) the hydro-lyases that use protein-bound Fe-S clusters in catalysis; and (3) hydro-lyases that appear to require a metal such as iron for catalysis, but lack a Fe-S cluster. The latter group of enzymes needs further study before they can be confidently established as a subclass.

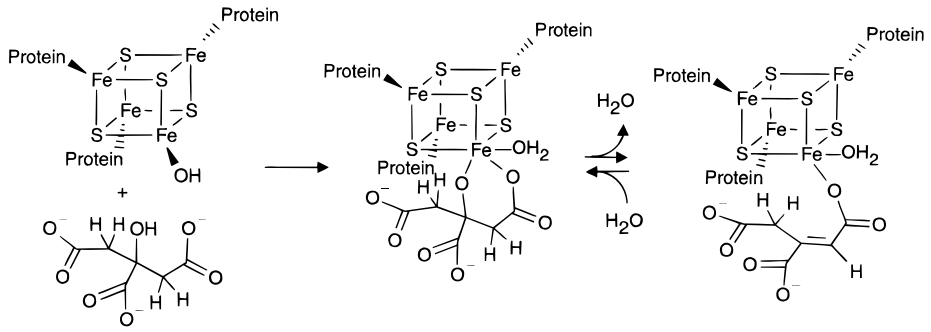


1 2 (1)

We direct our attention in this review to those members of the second and third subclasses, but it will soon become evident to the reader that only a few of the hydro-lyases have been studied in sufficient detail to be able to place them with full confidence into one of these subclasses. Our aim in writing this review was to be comprehensive and point out some of the unsolved problems in the hydro-lyase class; therefore, we have not hesitated to discuss the understudied enzymes and to hazard a guess as to which subclass they may belong.

There are several criteria that are useful in establishing the presence of a Fe-S cluster in a protein including iron and sulfide analysis; optical, EPR, resonance Raman, MCD, and Mössbauer spectroscopies; and, to a certain extent, amino acid sequence patterns. In many Fe-S cluster-containing proteins some of the cysteine residues that ligand the cluster exist in the motif C-X_2-C or its extended form, C-X_2-C-X_2-C (where C represents a cysteine residue and X represents any other amino acid). There are enough proteins where such motifs exist and the cysteines in them are ligands to the protein's Fe-S cluster that the presence of such motifs is often taken as suggestive evidence of the presence of a Fe-S cluster.[8] While it can be helpful to use this motif as a guide in searching for Fe-S cluster-containing

Scheme 1

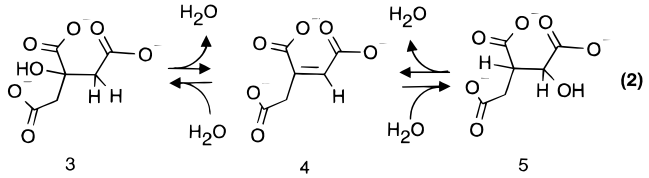


proteins and the cysteine ligands to their clusters, it should be used with caution because it is not an infallible guide.

Although the dehydration reaction depicted in reaction 1 appears to be chemically straightforward, the impressive catalytic burden borne by one member of the hydro-lyase class, mammalian fumarase, has recently been described in an elegant paper from Wolfenden's group.[24] Mammalian fumarase enhances the rate of the interconversion of malate and fumarate 3×10^{15}-fold over the uncatalyzed rate at pH 6.8 and 37 °C by stabilizing the transition state structure (ΔG_{tx}) by at least 30 kcal/mol. The degree of transition-state stabilization provided by this enzyme is much greater than that observed in other enzymes catalyzing C−H bond cleavage—even those working at a diffusion controlled rate (e.g., triose phosphate isomerase). Orotodine monophosphate decarboxylase, which catalyzes C−C bond cleavage, is the only enzyme known to contribute a greater degree of transition state stabilization than fumarase. Although mammalian fumarase does not contain a Fe-S cluster, members of the subclass of hydrolyases that do may contribute a similar degree of transition state stabilization—emphasizing the remarkable nature of the enzymes in this class.

A. Aconitase

Aconitase [EC 4.2.1.3] catalyzes reaction 2 in which the TCA cycle intermediates citrate **3** and isocitrate **5** are interconverted via the intermediate aconitate **4**. Mammalian mitochondrial aconitase contains a $[4Fe\text{-}4S]^{2+,+}$ cluster (a discussion of the meaning of this term is given in ref 23). This enzyme has been more extensively studied than other Fe-S cluster-containing members of the hydro-lyase class. An up to date review on aconitase can be found in a separate review in this issue of *Chemical Reviews*.[25] The current understanding of the mechanism of aconitase and the role of the Fe-S cluster in it will be briefly recapitulated here because aconitase will be used as a model for other hydro-lyases that contain Fe-S clusters. We will refer to this model as the aconitase paradigm.



(2)

3 4 5

The important features of the paradigm are as follows:

(1) Aconitase contains in its native state a $[4Fe\text{-}4S]^{2+,+}$ cluster.

(2) The clusterless apoprotein is unable to catalyze reaction 2.

(3) Only three of the iron atoms in the cluster have ligands from the protein (the ligands are sulfur from C_{358}, C_{431}, and C_{434}; the motif is C-X_{75}-C-X_2-C).

(4) In the substrate-free form, the ligands for the fourth iron atom are OH^- along with the bridging sulfides of the Fe-S cluster.

(5) The fourth iron is exposed to the substrate binding cavity, and on binding the substrate (citrate or isocitrate) the hydroxyl group and one carboxyl group become ligands to the fourth iron.

(6) The Fe-S cluster participates in catalysis by acting as a Lewis acid to activate the hydroxyl group of the substrate for elimination.

(7) When one iron is lost from the cluster, enzymatic activity is reduced over 100-fold and a reasonably stable [3Fe-4S] cluster is formed.

(8) The largely inactive enzyme with a [3Fe-4S] cluster can upon reduction pick up a fourth iron from solution, re-form a [4Fe-4S] cluster, and regain full activity.

(9) A [4Fe-4S] cluster can, to a limited extent, form on the inactive apoprotein in the presence of iron, sulfide, and thiol-containing compounds and thereby activity is regained.

(10) aconitase is strongly inhibited by nitro analogs of its natural substrates, indicating the reaction takes place by an $E1_{cb}$ mechanism.

Scheme 1 outlines some of the events in the reaction catalyzed by aconitase (the reader is referred to the review on aconitase included in this volume of *Chemical Reviews* for a more detailed discussion of its mechanism[25]).

As the aconitase paradigm developed, the possibility came forward that other Fe-S cluster containing hydro-lyases might exist, and their clusters might resemble in form and function the Fe-S cluster of aconitase. This possibility has been tested and verified in a few cases, so the aconitase paradigm may have broad applicability to Fe-S cluster-containing members of the hydro-lyase class.

Before the paradigm can be used, one must identify the members of the hydro-lyase class that contain Fe-S clusters. This is no small problem because our current knowledge of the bioinorganic aspects of many of them is little more than that they are activated in the presence of ferrous ions and thiols—

roughly equivalent to what was known about aconitase 40 years ago.[26] It is tempting to speculate that such members of the hydro-lyase class will eventually be shown to contain Fe-S clusters and that their clusters will be found to participate in the enzyme's catalytic function. However, some of the work reviewed here suggests that such speculation should be applied with caution because there is now evidence that not all hydro-lyases that require ferrous ions for activity contain a Fe-S cluster.

As the mechanistic studies on aconitase were nearing completion, our knowledge of this enzyme seemed to be settling into a comfortable new steady state. In the meantime, important developments were taking place in a seemingly distantly related field, the regulation of iron uptake and transport in mammals. Then in one of those unexpected discoveries referred to in the introduction, these two fields were found to overlap, thereby drastically expanding our understanding of the roles performed by aconitase. The major events in this discovery are recounted below.

It had been known for several years that ferrous ion levels control the expression of the iron storage protein, ferritin, and the iron uptake protein, transferrin receptor.[27] A protein was identified that modulates this control, and the protein was named the iron-responsive element binding protein, commonly abbreviated IRE-BP. (Recently the name of this protein has been changed to the iron regulatory protein, which is abbreviated IRP or IRP1; however, since this is a historical account, the original term IRE-BP will be used in this review.[28,29]) The protein was so called because it had been found to bind to iron regulatory elements, commonly abbreviated IREs, which are *cis*-acting elements found in the m-RNA of ferritin and transferrin receptor.[30,31] These elements form stem-loop structures to which the IRE-BP binds when the iron content of the cells is low.

The IREs in ferritin m-RNA are in the untranslated region near the 5′ end; therefore, when the IRE-BP binds to ferritin m-RNA its translation is blocked. The IREs in transferrin receptor m-RNA are in the untranslated region near the 3′ end; therefore, when the IRE-BP binds transferrin receptor m-RNA, its half-life is extended, which increases the translation of transferrin m-RNA.[32] When the iron content is high, IRE-BP does not bind to IREs, the translation of ferritin m-RNA is unimpeded, and the translation of transferrin receptor m-RNA declines because its half-life is shortened. When the iron content is low, IRE-BP binds to IREs, the translation of transferrin receptor m-RNA increases while the translation of ferritin m-RNA is impeded. Thus the overall effect of this regulatory system is to decrease iron uptake and increase iron storage when the cellular iron content is high, and to increase iron uptake and decrease iron storage when the iron content is low.

IRE-BP was sequenced, and found to have significant homology with mitochondrial aconitase (30% identity; 57% similarity).[33] Next, it was discovered that IRE-BP exhibited aconitase activity.[34] Finally, IRE-BP and cytoplasmic aconitase were found to be the same protein![35,36] A protein regulating iron uptake had been found to contain a Fe-S cluster. The three cysteines known from the crystal structure of mitochondrial aconitase to ligand its Fe-S cluster are conserved in cytoplasmic aconitase/IRE-BP as C_{385}, C_{451}, and C_{454} (motif: C-X_{65}-C-X_2-C) along with the catalytic active site bases.[37] Presumably these three cysteines are ligands to the Fe-S cluster of cytoplasmic aconitase/IRE-BP.

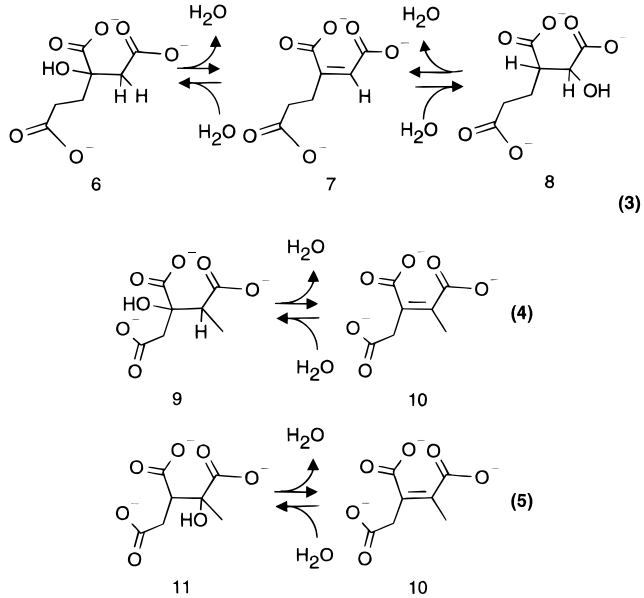
The Fe-S cluster participates in the regulation of iron uptake as follows. When the iron concentration is high, a Fe-S cluster is formed on this protein, which blocks its interaction with IREs. With a Fe-S cluster, the protein has aconitase activity. When the iron concentration is low, the Fe-S cluster and enzymatic activity are lost. The clusterless protein binds to the IREs.[38,39] An up to date review of IRE-BP is included in the review of aconitase in this issue of *Chemical Reviews*.[25]

In the transition from high to low iron, it is not clear how the cluster is lost (spontaneous dissociation, through NO or a NO derivative, oxidation by O_2, or O_2^-?). Nevertheless, the most logical conclusion is that this protein cycles between holo and apo states according to the iron status of the cell, and the ratio of holo- to apoprotein appears to be the way mammalian cells monitor and respond to iron levels.

The paradigm presented by aconitase/IRE-BP consists in part of an iron and sulfide dependent protein containing a Fe-S cluster that performs, in the case of cytoplasmic aconitase, the dual roles of catalysis and regulation. It will be interesting to find out how frequently nature has used Fe-S clusters to perform multiple roles.

B. Homoaconitase, Methylcitrate Dehydratase, and 2-Methylisocitrate Dehydratase

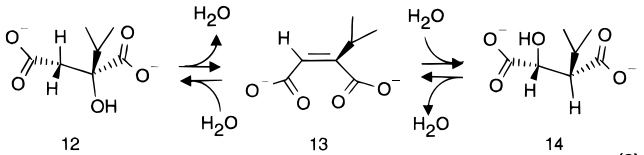
Homoaconitase [EC 4.2.1.36], methylcitrate dehydratase [EC 4.2.1.79], and methylisocitrate dehydratase are three members of the hydro-lyase class whose substrates closely resemble those of aconitase. These enzyme catalyze, respectively, reactions 3, 4, and 5. Homoaconitase functions in the fungal pathway for the synthesis of lysine. Yeast homoaconitase has been partially characterized and found to be inhibited by iron chelators suggesting that it may contain a Fe-S cluster.[40]



Methylcitrate dehydratase and methylisocitrate dehydratase from *Yallowia lipolytica* function in propionyl-CoA oxidation. Neither methylcitrate dehydratase nor 2-methylisocitrate dehydratase catalyzes the reaction catalyzed by the other although the dehydrated product **10** of both enzymes is the same compound. These enzymes have not been well characterized, but neither appears to contain a Fe-S cluster.[41,42]

C. Isopropylmalate Isomerase

3-Isopropylmalate isomerase [EC 1.2.1.33] functions in the leucine arm of the branched-chain amino acid biosynthetic pathway where it catalyzes reaction 6, the interconversion of **12** and **14** by way of the unsaturated intermediate **13**. The reaction is similar to that catalyzed by aconitase.



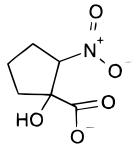
12 13 14 (6)

Isopropylmalate isomerase has been purified aerobically from *Saccharomyces cerevisiae* and has been found to contain 0.8 mol of iron and sulfide per mole of protein. As isolated, the enzyme is EPR silent, but on reduction with sodium dithionite a broad axial signal appears with $g_{av} = 1.96$ ($S = {}^1/_2$). When **12** is added to the reduced enzyme, the EPR signal changes dramatically with the formation of two species, one with $g_{av} = 1.90$ (major species) and one with $g_{av} = 1.92$ (minor species). When this experiment is conducted in ^{17}O water, the EPR signal is broadened. On oxidation of the native enzyme with $K_3Fe(CN)_6$ an EPR signal appears near $g = 2.01$ ($S = {}^1/_2$).[43] These results are consistent with this enzyme containing a $[4Fe\text{-}4S]^{2+,+}$ cluster that performs a function in catalysis similar to that performed by the Fe-S cluster of aconitase. The broadened EPR signal in ^{17}O water indicates direct coordination of water or hydroxylated substrate (or both) to an iron atom in the cluster.

Yeast isopropylmalate isomerase readily loses one iron in air to form a $[3Fe\text{-}4S]^+$ cluster, which is not as stable as the $[3Fe\text{-}4S]^+$ cluster of mammalian aconitase, and degrades further to the apoprotein. It is likely that a substantial fraction of this protein lost its Fe-S cluster during aerobic purification, accounting for the less than expected amount of iron and sulfide found in the purified protein.

The sequence of *Mucor circinelloides* isopropylmalate isomerase is homologous to mammalian aconitase (23% identity and 51% similarity). All three cysteines known from the crystal structure of mitochondrial aconitase to ligand its Fe-S cluster are preserved in isopropylmalate isomerase (C_{400}, C_{460}, and C_{463}; motif: C- X_{59}-C-X_2-C) and presumably are the ligands to its Fe-S cluster.[44]

Nitro analogs of isopropylmalate are potent reversible inhibitors of this enzyme, suggesting the reaction occurs by an $E1_{cb}$ mechanism (see the section on FumA and FumB for a more detailed discussion of the inhibition of hydro-lyases by nitronate analogs of their substrates). The nitro analog **15** has significant herbicidal activity.[45]



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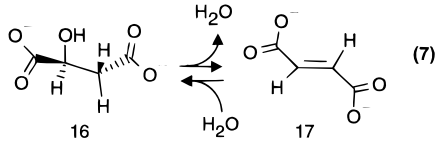
D. Fumarase A and B

Fumarase [EC 4.2.1.2] catalyzes reaction 7, the reversible dehydration of (2*S*)-malate **16** to fumarate **17**. This reaction is part of the tricarboxylic acid cycle. It is catalyzed in mammals by an intensively studied nonmetalloprotein that works at close to the diffusion control rate.[46]



16 17 (7)

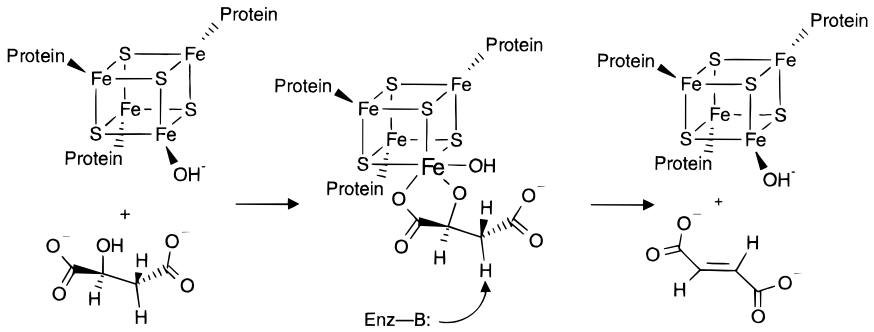
A few years ago it was discovered that *Escherichia coli* contains three fumarase genes designated *fumA*, *fumB*, and *fumC*. The amino acid sequences of FumA and FumB are homologous with each other (90% identity) but have little homology to FumC or the mammalian fumarases.[47,48] There is considerable homology between FumC and the mammalian fumarases (60% identity).[49,50]

The activity of FumA and FumB is unstable in the presence of O_2, so anaerobic conditions were used in their isolation. Both enzymes have a brown color, contain close to four iron and four sulfide atoms per monomer, and have an optical spectrum characteristic of proteins with [4Fe-4S] clusters. In the native state these proteins are EPR silent. FumA can be reduced (redox potential ≈ -480 mV) to an EPR active state exhibiting a rhombic spectrum at high fields (g values = 2.03, 1.94, and 1.88) and a broad peak at $g = 5.4$. Upon addition of substrate, the high-field signal shifts (g values = 2.035, 1.92, and 1.815) and the total spins increases by 8-fold, while the $g = 5.4$ signal disappears. Similar results have been obtained with FumB. These results indicate that FumA and FumB contain $[4Fe\text{-}4S]^{2+,+}$ clusters.[51−55] Apparently, the reduced clusters of the substrate-free enzymes exist in a mixture of $S = {}^1/_2$ and $S = {}^3/_2$ ground states, but shift to a $S = {}^1/_2$ ground state in the presence of substrate. The activity of FumA is decreased over 10-fold by photoreduction, which lowers the V_{max} and increases the K_m for malate.

There are nine conserved cysteines in FumA and FumB (C_{104}, C_{181}, C_{223}, C_{309}, C_{317}, C_{473}, C_{501}, C_{542}, and C_{545}; motif: C-X_{76}-C-X_{41}-C-X_{85}-C-X_{59}-C-X_7-C-X_{155}-C-X_{27}-C-X_2-C).[47,48] Since the sulfur from cysteines in the motif C-X_2-C are often found to be ligands in Fe-S cluster-containing proteins, C_{542} and C_{545} seem likely candidates for ligands to the clusters of FumA and FumB.

The Fe-S clusters of FumA and FumB can be oxidized by O_2 and $K_3Fe(CN)_6$ to generate inactive forms of these enzymes that have axial EPR signals at $g = 2.02$, consistent with the presence of $[3Fe\text{-}4S]^+$

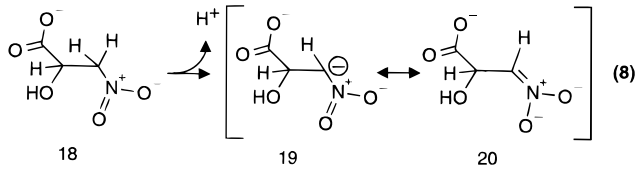
Scheme 2



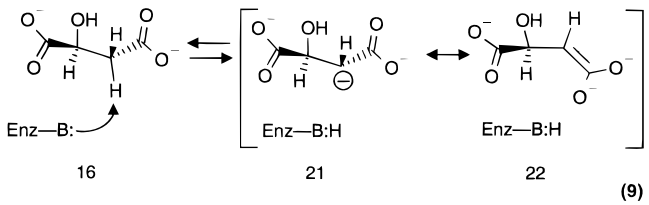
clusters. The $[3Fe\text{-}4S]^+$ clusters of FumA and FumB are not nearly as stable as the $[3Fe\text{-}4S]^+$ cluster of mammalian aconitase and readily lose additional iron, but they are stable enough to be studied by EPR.[51] The activity of partially inactivated FumA containing a mixture of [3Fe-4S] and [4Fe-4S] clusters can be restored by incubation with iron and a thiol-containing solution.[55] FumA and FumB are rapidly inhibited by O_2^- (see discussion in section IV of this review).

These results are all consistent with the Fe-S cluster of FumA and FumB performing a function in catalysis as a Lewis acid as outlined in Scheme 2. Since (2*S*)-malate is the only enantiomer of malate that is a substrate for FumA and the (3*R*) proton is removed in the reaction, this enzyme must catalyze an antiperiplanar elimination of the elements of water from malate.[56] This is identical to the stereochemistry of the reaction catalyzed by mammalian fumarase.

Like aconitase, isopropylmalate isomerase, and mammalian fumarase, FumA is strongly inhibited by the nitronate analog 2-hydroxy-3-nitropropionate (**18**) of one of its natural substrates, malate **16**, when the nitronate is in the dianionic form **19** and **20** shown in reaction 8.[56] The inhibition by the dianionic form



of **18** most likely means the reaction catalyzed by FumA occurs by way of an $E1_{cb}$ mechanism as shown in reaction 9 in which the carbanion is a distinct intermediate.



The strong binding of the dianionic form of 2-hydroxy-3-nitropropionate **19** and **20** is thought to be due to its resemblance to the carbanion reaction intermediate **21** and **22**.

It has been shown that (2*S*,3*R*)-[3-^{2}H]malate does not give rise to a deuterium isotope effect in the reaction catalyzed by FumA, indicating the formation of the carbanion intermediate in the reaction is not the rate-limiting step.[56] The pK_as of the catalytic groups of both mammalian fumarase and FumA have been determined and found to differ. Nonetheless, except for the way the hydroxyl group is activated for elimination, the fundamental steps in the underlying mechanism of these two enzymes appear to be similar. This similarity could extend to all members of the hydro-lyase family (both with and without Fe-S clusters) that catalyze a dehydration between the carbons α and β to a carboxyl group, the hydroxyl group being removed from the β carbon. All such enzymes appear to form a carbanionic intermediate in a non-rate-determining step in the early stages of the reaction they catalyze, and remove the elements of water from substrate in an antiperiplanar fashion.[57]

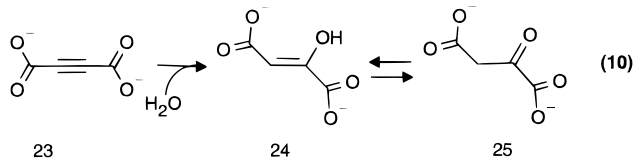
Table 1. Kinetic Constants of *E. coli* Fumarase A and Mammalian Fumarase

enzyme	substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\ M^{-1}$)
E. coli fumarase A	fumarate	0.6	3100	5×10^6
E. coli fumarase A	malate	0.7	670	9×10^5
mammalian fumarase[a]	fumarate	0.005	200	4×10^7
mammalian fumarase[a]	malate	0.025	225	9×10^6

[a] Hill, R. L.; Teipel J. W. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. 5, p 539. Hill, R. L.; Bradshaw, R. A. In *Methods In Enzymology*; Colowick, S. P., Kaplan, N. O., Eds.; Academic Press: New York, 1969; Vol. XIII, p 91. Frieden, C.; Alberty, R. A. *J. Biol. Chem.* **1955**, *212*, 859. (Reprinted from ref 56. Copyright 1994 Academic Press.)

FumA has the highest k_{cat}/K_m yet measured for a Fe-S cluster containing member of the hydro-lyase class. The kinetic constants of FumA and mammalian fumarase, an enzyme whose turnover is known to be near the diffusion control rate,[46] are compared in Table 1. These constants demonstrate that Fe-S cluster containing hydro-lyases can be highly efficient catalysts.

In addition to catalyzing the hydration of fumarate, FumA catalyzes the hydration of acetylenedicarboxylate **23** to enol oxalacetate **24**, and the tautomerization of enol oxalacetate to oxalacetate **25** as shown in reaction 10. It catalyzes the hydration of acetylene dicarboxylate at nearly the same rate as it catalyzes the hydration of fumarate.[56,58] Recently an acetylene hydratase has been reported in *Pelobacter acetylenicus* that catalyzes a closely analogous reaction, the hydration of acetylene to acetaldehyde. This enzyme is a tungsten Fe-S protein.[59]

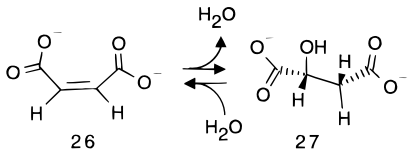


(10)

Reaction 10 has been used to follow the transfer of ^{18}O from (2*S*)-[2-^{18}O]malate and ^{2}H from (2*S*, 3*R*)-[3-^{2}H]malate to acetylenedicarboxylate. It was found that at infinite concentration of acetylenedicarboxylate 33% of the ^{18}O and close to 100% of the ^{2}H would be transferred from the carbon skeleton of malate to that of acetylenedicarboxylate. This is one of the few, if not only, demonstrations of ^{18}O transfer by an enzyme from one carbon skeleton to another. From the ^{18}O transfer results and the rate constants in Table 1 one can calculate that the rate constant for the dissociation of the hydroxyl group from FumA following extraction from malate is between 1×10^4 and 8×10^5 s^{-1}.[58] This rate is of interest because it probably is close if not equivalent to the rate at which the oxygen of the abstracted hydroxyl group exchanges off the iron in the cluster. It was pleasing to find that this rate is of the same order of magnitude as the measured exchange rates of water off monomer iron with five other anion ligands.

E. Maleate Hydratase and Dimethylmaleate Hydratase

Maleate hydratase [EC 1.2.1.31] has been purified from rabbit kidneys and *Arthrobacter* sp. strain MCI2612. It carries out reaction 11, the hydration of maleate **26** to (2*R*)-malate **27**. The native kidney enzyme appears to be a monomer with a mass of 68 kDa, whereas the native *Arthrobacter* enzyme appears to be a heterodimer with a mass of 90 kDa comprised of 58 and 28 kDa subunits.

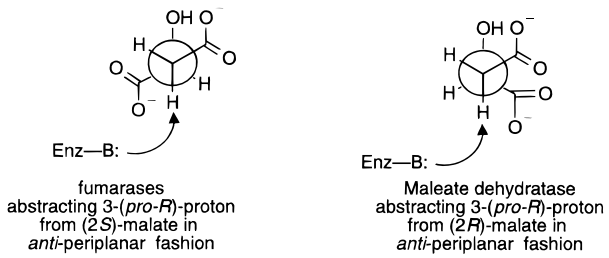


(11)

The physiologically relevant direction for this reaction in both kidneys and bacteria, where it performs a catabolic role, is from **26** to **27**. The malate formed is subsequently oxidized to oxalacetate by a (2*R*)-2-hydroxy acid dehydrogenase. Kidney maleate hydratase is thought to perform a role in the control of renal activity and has an absolute requirement for chloride ions.

Maleate hydratase and fumarase accept as substrates the opposite enantiomers of malate; respectively **16** and **27**. The dehydrated products of maleate hydratase and fumarase are themselves opposite stereoisomers, (*E*)- and (*Z*)-butenedioic acid, respectively. As previously mentioned, the fumarases remove the 3-*pro*-*R* proton of malate during dehydration by an antiperiplanar elimination.[56] The dehydration catalyzed by maleate hydratase also takes place by an antiperiplanar elimination in which the 3-*pro*-*R* proton is removed. Scheme 3 depicts the relative stereochemistries.[60]

Scheme 3



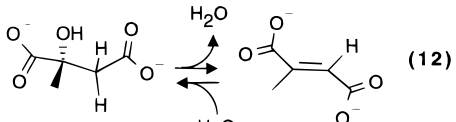
Both the kidney and *Arthrobacter* enzymes are inhibited by chelators and are unstable in air with the kidney enzyme being the more labile. Both enzymes can be reactivated upon incubation in solutions containing ferrous ions and thiols, with the kidney enzyme requiring Na_2S as well for maximal reactivation.[61,62] These observations suggest that this enzyme from both sources may contain a Fe-S cluster.

Reactivated kidney maleate hydratase contains 4−5 mol of iron and 4−6 mol of sulfide per mole of enzyme. The optical spectrum of the reactivated enzyme has an absorbance peak near 415 nm, is typical of a protein with a [4Fe-4S] cluster, and is bleached on the addition of dithionite. However, no EPR spectrum characteristic of a reduced $S = {}^1/_2$ [4Fe-4S] cluster or [2Fe-2S] cluster was found. This could be due to an instability in the reduced cluster, or the reduced cluster being a $S = {}^3/_2$ or ${}^5/_2$ system. An EPR spectrum characteristic of a $[3Fe\text{-}4S]^+$ cluster was found in purified samples of the protein, with much stronger signals being detected from reactivated protein that had then been subsequently exposed to air for a short time (and thereby inactivated).[62]

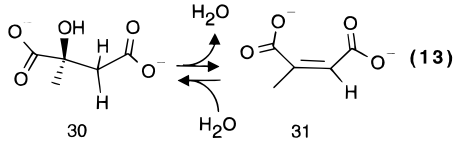
Dimethylmaleate hydratase [EC 4.2.1.85] has been partially purified from *Clostridium barkeri*.[63] The enzyme is unstable in the presence of oxygen. Following inactivation by oxygen, the enzyme can be reactivated in the presence of ferrous ions along with a thiol-reducing agent. These observations suggest this enzyme may contain a Fe-S cluster.

F. Mesaconase and Citraconase

Mesaconase [EC 1.2.1.34] and citraconase [EC 1.2.1.35] carry out reactions 12 and 13 using as substrates the (2*S*)- **28** and (2*R*)-methylmalate **30**.



(12)



(13)

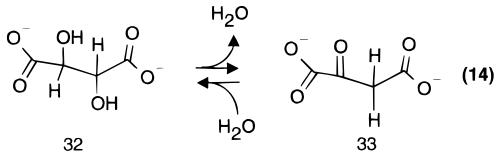
These two enzymes catalyze reactions that bear the same stereochemical relationship to each other as those catalyzed by fumarase and maleate hydratase. It is highly likely that both enzymes catalyze elimination in an antiperiplanar fashion in which the

3-(*pro-R*) proton is removed from their respective substrates held by the enzymes in conformations analogous to those shown in Scheme 3.

Mesaconase has been partially purified from *Pseudomonas arvilla*,[64] *Clostridium tetanomorphum*,[65,66] an unknown aerobic bacterium,[67] and citraconase has been purified from a *Pseudomonas* species.[68] All four enzymes are inactivated in the presence of O_2, inhibited by chelators, and activated by incubation in solutions containing ferrous ions and thiol-containing compounds. These observations suggest that these enzymes may contain catalytically active Fe-S clusters. Mesaconase from all three sources is also a catalyst of the fumarase reaction.[64,67]

G. Tartrate Dehydratase

Isozymes of tartrate dehydratase catalyze the dehydration of L-(+)-(2*R*,3*R*)-tartrate **32**, D-(−)-(2*S*,3*S*)-tartrate **32**, and *meso*-(2*R*,3*S*)-tartrate **32** to form oxalacetate **33** as shown in reaction 14.

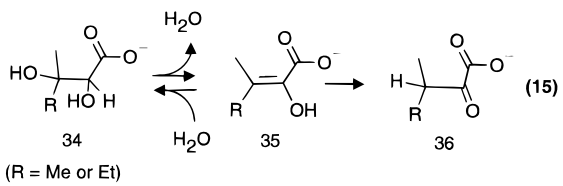


L-(+)-Tartrate dehydratase from *Pseudomonas putida* has been purified to near homogeneity using tandem dye columns. The enzyme is an $\alpha_2\beta_2$ heterotetramer with subunits of 23 and 27 kDa.[69] It loses activity rapidly in air and can be reactivated as much as 100-fold and stabilized in solutions containing ferrous ions and thiols.[70,71] The reactivated enzyme contains approximately four iron atoms and four acid-labile sulfides per mole of tetramer. If the reactivated enzyme has a full complement of cluster (which is questionable) and assuming symmetry, these results suggest that this enzyme may contain two [2Fe-2S] clusters or one [4Fe-4S] cluster per tetramer.[69] Unfortunately no optical or EPR spectra of this enzyme are available, and it is not known if the reactivation was partial or complete.

D-(+)-Tartrate dehydratase from *Rhodopseudomonas sphaeroides* is very stable in the presence of O_2 and does not require iron.[72] D-(+)-Tartrate dehydratase from a *Pseudomonas* species requires either iron or cobalt for activity but does not require a thiol compound for activation. It seems doubtful that either of these enzymes contains a Fe-S cluster.[73]

H. Dihydroxy-acid Dehydratase

Dihydroxy-acid dehydratase catalyzes reaction 15, the third reaction in the branched-chain amino acid biosynthetic pathway. This enzyme has been isolated



from *E. coli* and spinach, and some aspects of this enzyme have been reviewed previously.[74] The activity of the *E. coli* enzyme rapidly declines in the presence of O_2 so anaerobic purification is required to maintain activity.

The pure enzyme contains 2−3 mol of iron and 2−3 mol of sulfide per mole of monomer. It is brown in color and has an optical spectrum similar to proteins with [4Fe-4S] clusters including a peak at 410 nm. The enzyme is EPR silent as isolated, but exhibits a complex EPR spectrum on reduction with dithionite whose major features are consistent with this enzyme containing a $[4Fe\text{-}4S]^{2+,+}$ cluster predominantly in the $S = {}^3/_2$ spin state, with minor amounts in the $S = {}^1/_2$ and $S = {}^5/_2$ spin states. Resonance Raman studies and low-temperature MCD spectra also support this conclusion.[75]

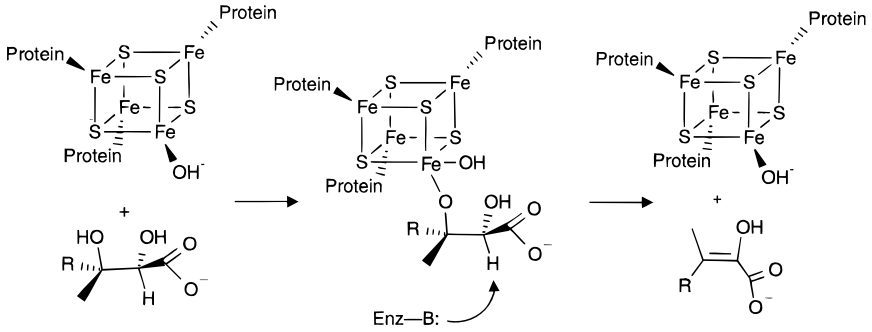
The low iron and sulfur content of the purified enzyme (compared to what is expected if the enzyme contains one [4Fe-4S] cluster/monomer) is due to cluster loss during isolation, even though it is purified anaerobically. The activity of this enzyme is lost when iron is removed from the cluster, indicating that the presence of the intact cluster is essential for catalysis. The $[3Fe\text{-}4S]^+$ cluster form of this enzyme exists in both the cubane and linear forms, and is very unstable.[75] *E. coli* dihydroxy-acid dehydratase is inactivated by cluster destruction in the presence of O_2 and much more rapidly in the presence of O_2^-.[76]

The resonance Raman spectrum of this enzyme indicates that the cluster has a non-cysteinyl ligand coordinated to one of the iron atoms. Its totally symmetric bridging Fe−S stretching mode of the [4Fe-4S] cubane core occurs at 343 cm^{-1}, which is in the range normally found for proteins with $[4Fe\text{-}4S]^{2+,+}$ clusters that have incomplete cysteinyl coordination.[75] This suggests that the cluster ligation state could be similar to that of mammalian aconitase in which only three of the iron atoms in the cluster have ligands from the protein while the ligand for the fourth iron atom is OH^-. It seems reasonable to postulate that the iron not liganded by a cysteine residue in dihydroxy-acid dehydratase may also have as a ligand OH^- from solvent.

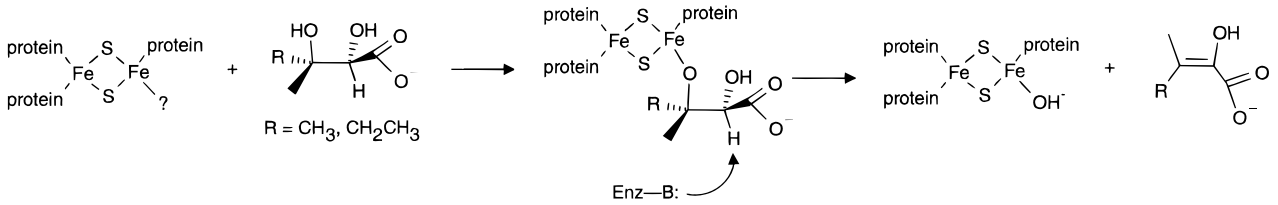
The *ilvD* genes encoding dihydroxy-acid dehydratase from *E. coli* and *Lactobacillus lactis* have been sequenced, and a homologous gene has been found in *Haemophilus influenzae*.[77,78] The amino acid sequences of the enzyme from *L. lactis* and the putative enzyme from *H. influenzae* share, respectively, 40% and 78% identity and 61% and 89% similarity with the enzyme from *E. coli*. Of the 12 cysteines in *E. coli* dihydroxy-acid dehydratase (C_{112}, C_{118}, C_{122}, C_{189}, C_{192}, C_{195}, C_{206}, C_{312}, C_{395}, C_{408}, C_{434}, and C_{508}; motif: $C\text{-}X_5\text{-}C\text{-}X_3\text{-}C\text{-}X_{66}\text{-}C\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_{10}\text{-}C\text{-}X_{105}\text{-}C\text{-}X_{82}\text{-}C\text{-}X_{12}\text{-}C\text{-}X_{82}\text{-}C\text{-}X_{25}\text{-}C$) all but one, C_{312}, are conserved in the putative *H. influenzae* enzyme, but only three, C_{122}, C_{195}, C_{508}, are conserved in the *L. lactis* enzyme. This low number of conserved cysteines (25%) is surprising given the otherwise 40% identity between these two proteins.

If we assume that the *L. lactis* enzyme has a [4Fe-4S] cluster similar to that of *E. coli* enzyme, we would expect there to be three cysteines that would donate ligands to the Fe-S clusters of both of these proteins. It seems reasonable to expect that the liganding cysteines from both proteins would be the three that

Scheme 4



Scheme 5



are conserved between them. If this is the case, then the motif C-X_2-C-X_2-C in *E. coli* dihydroxy-acid dehydratase only contributes one sulfur ligand to the Fe-S cluster.

Scheme 4 illustrates diagrammatically the postulated function of the cluster of *E. coli* dihydroxy-acid dehydratase, acting as a Lewis acid to facilitate the elimination of the 3-hydroxyl group of the substrate. Neither the carboxyl group nor the 2-hydroxyl group of the substrate is depicted in Scheme 4 as a ligand for the cluster, but it seems likely that one or both could be.

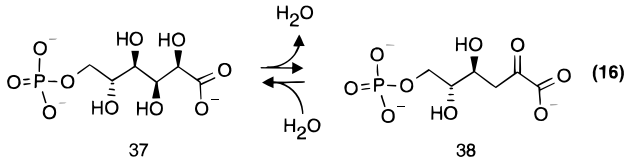
Spinach dihydroxy-acid dehydratase is stable in air and has been isolated under aerobic conditions. It is a homodimer containing 2 mol of iron and 2 mol of sulfide per mole of monomer. The protein is brown in color and has an optical spectrum characteristic of proteins with [2Fe-2S] clusters. The isolated protein is EPR silent but on reduction exhibits an EPR spectrum ($g_{av} = 1.91$), which is characteristic of Rieske proteins. However, the redox potential (−470 mV) is closer to that of a typical ferredoxin type [2Fe-2S] cluster. These results suggest that the spinach dihydroxy-acid dehydratase contains a [2Fe-2S]$^{2+,+}$ cluster liganded with cysteines and at least one negatively charged non-cysteinyl ligand.[79]

Addition of substrate to the reduced spinach enzyme dramatically effects the EPR spectrum. The turnover rate of the reduced enzyme is about one-sixth that of the native enzyme. These results suggest that the substrate interacts directly with the Fe-S cluster of this enzyme, implying that the cluster performs a role in catalysis as a Lewis acid as outlined in Scheme 5.[79]

Spinach dihydroxy-acid dehydratase is the only member of the Fe-S cluster containing subclass of hydro-lyases known to contain a [2Fe-2S] cluster. This enzyme is the only one known to not be inactivated by O_2 or O_2^-. It is not known if there is a cause and effect relationship between these two characteristics (see section IV of this review for a detailed discussion of the effect of O_2^- on hydro-lyases with Fe-S clusters, and a discussion of possible reasons for the resistance of the spinach enzyme to O_2 and O_2^-).

I. Phosphogluconate Dehydratase

6-Phosphogluconate dehydratase [EC 1.2.1.12] catalyzes reaction 16, the dehydration of **37** to a 2-enol followed by its tautomerization to a 2-keto carboxylic acid **38**.[80] This reaction is very similar to the reaction catalyzed by dihydroxy-acid dehydratase.

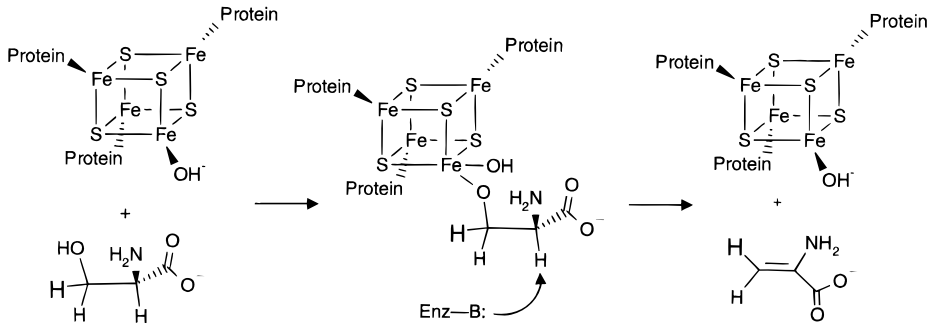


Many years ago, it was found that 6-phosphogluconate dehydratase from *Pseudomonas fluorescens* required the presence of ferrous ions and a thiol for activity.[81] Later, the 6-phosphogluconate dehydratase from *Zymomonas mobilus* was reported to have four iron atoms and one manganese atom per subunit and to have an optical absorbance spectrum typical of proteins with [4Fe-4S] clusters.[82,83] The *Z. mobilus* enzyme is inactivated by 2 equiv of ferricyanide and by the chelator 1,10-phenanthroline. These results are consistent with the native form of this enzyme from these two organisms containing Fe-S clusters that may participate in catalysis. The activity of *E. coli* 6-phosphogluconate dehydratase is stabilized by fluoride, which has also been shown to stabilize *E. coli* dihydroxy-acid dehydratase, FumA, and FumB; presumably by stabilizing their Fe-S clusters (perhaps by becoming a ligand to the cluster).[55,84] *Z. mobilus* and *E. coli* 6-phosphogluconate are inactivated by H_2O_2 and O_2^-.[83,84] As described previously, this behavior is shared by other [4Fe-4S] cluster-containing members of the hydro-lyase class.

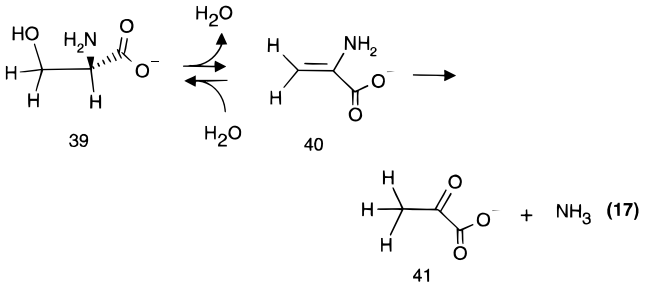
J. Serine Dehydratase

The almost ubiquitous existence of L-serine [EC 4.2.1.13] and L-threonine dehydratases [EC 4.2.1.16]

Scheme 6



has been known for many years.[85] Serine dehydratase catalyzes the dehydration of L-serine **39** to form pyruvate **41** and ammonia via an eneamine intermediate **40** as shown in reaction 17.



(17)

Until recently, all such dehydratases were assumed to be metal-free enzymes containing pyridoxal phosphate as a cofactor. The first hint (ignored at the time) that this might not be the case came almost 25 years ago when it was found that the L-serine dehydratase purified from the anaerobe *Clostridium acidiurici* was inactive, but could be activated in solutions containing ferrous ions and dithiothreitol.[86] L-serine dehydratase from *E. coli* also displays this behavior.[87,88] The significance of this finding was not appreciated until the aconitase paradigm emerged. Since then, several bacterial serine dehydratases have been found to contain Fe-S clusters.[11,89]

The first example was the serine dehydratase from the strict anaerobe *Peptostreptococcus asaccharolyticus*. The enzyme (1) is not inhibited by $NaBH_4$ or phenylhydrazine, (2) lacks detectable pyridoxal phosphate, (3) has a yellow-brown color and an absorbance maximum at 420 nm that is characteristic of a [4Fe-4S] cluster, (4) contains about 4 mol of iron and 5 mol of sulfide per heterodimer (55 kDa), (5) is inactivated in air, (6) in the inactive form can be reactivated with Fe^{2+} and dithiothreitol, and (7) has been shown by EPR spectroscopy (when the enzyme is isolated under anaerobic conditions) to contain a $[3Fe\text{-}4S]^+$ cluster.[90] Upon incubation in air or in $Fe(CN)_6^{3+}$-containing solutions the $[3Fe\text{-}4S]^+$ cluster signal increases in intensity while the activity decreases. These results suggest the active enzyme contains a $[4Fe\text{-}4S]^{2+,+}$ cluster. This enzyme is an $\alpha_2\beta_2$ tetramer so it will be important to find which subunits provides ligands for the cluster.

Attempts to observe an EPR signal characteristic of a $[4Fe\text{-}4S]^+$ cluster by reducing the enzyme with sodium dithionite or by photoreduction were not successful; apparently the cluster either was not reduced or was not stable on reduction. The Fe-S cluster appears to be near the serine binding site because the presence of serine effects the EPR spectra of the $[3Fe\text{-}4S]^+$ cluster (even though this form of the enzyme is inactive), and serine greatly retards the inactivation of the enzyme in air.[90]

By using the aconitase paradigm, these results are consistent with the Fe-S cluster in this enzyme catalyzing the dehydration of serine as indicated in Scheme 6.[11]

In addition to the enzyme from *P. asaccharolyticus*, an enzyme with similar properties has been isolated from the anaerobe *Clostridium propionicum*.[89] This enzyme is also an $\alpha_2\beta_2$ tetramer. *C. propionicum* serine dehydratase is considerably more sensitive to air than the enzyme from *P. asaccharolyticus*. There is some homology between the N-terminal amino acid sequences of the higher molecular weight α subunits and extensive homology between the N-terminal amino acid sequences of the smaller molecular weight β subunits of *C. propionicum* and *P. asaccharolyticus* serine dehydratases. Although the complete sequence of the subunits of these two enzymes has not been determined, these two proteins appear to be related.

Recently the *sdaA* and *sdaB* genes encoding L-serine dehydratase 1 and 2 of *E. coli* have been cloned and sequenced.[91,92] The amino acid sequences predicted for the proteins encoded by these two genes are 77% identical. *E. coli* serine dehydratase 1 and 2 do not require pyridoxal phosphate for activity nor do they contain the consensus sequence found in pyridoxal phosphate-dependent aminotransferases, (G)S(F)**K**(I)RG,[93] which contains the lysine whose ϵ-amino group characteristically enters into a Schiff base with pyridoxal phosphate when the enzyme is in the resting state. These proteins are activated in solutions containing ferrous ions and dithiothreitol.[94] It seems likely that the active form of the L-serine dehydratases from *E. coli* will be found to contain Fe-S clusters that participate in catalysis.

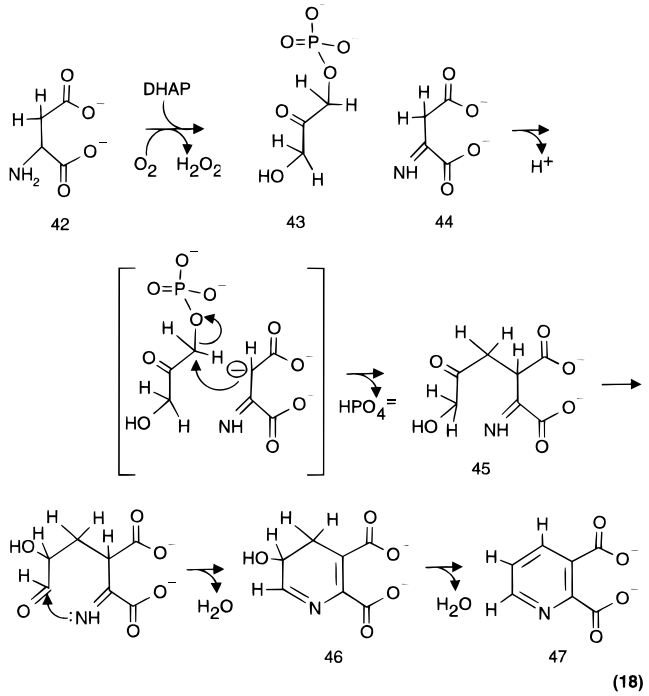
There are eight cysteines conserved between *E. coli* serine dehydratase 1 and 2 in the motif ($C\text{-}X_{108}\text{-}C\text{-}X_{48}\text{-}C\text{-}X_{7}\text{-}C\text{-}X_{18}\text{-}C\text{-}X_{14}\text{-}C\text{-}X_{20}\text{-}C\text{-}X_{110}\text{-}C$). The N-terminal amino acid sequences of the small subunit of *P. asaccharolyticus* and *C. propionicum* serine dehydratases have substantial sequence homology to the N-terminal region of *E. coli* serine dehydratase 1 and 2, suggesting that these proteins are related.

The L-threonine dehydratases characterized to date have been found to be typical pyridoxal phosphate enzymes that do not require iron. In contrast, all L-serine dehydratases characterized in detail to date

have been found to lack pyridoxal phosphate and require ferrous ions and dithiothreitol for activation. These seemingly similar amino acid dehydratases belong to two distinct mechanistic classes.

K. Quinolinate Synthetase

Quinolinate synthetase catalyzes reaction 18. This



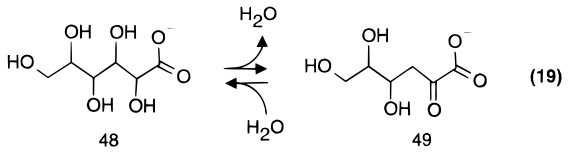
42 43 44 45 46 47 (18)

enzyme consists of two proteins that are referred to as A and B. B is a flavoprotein whose function is to oxidize aspartate **42** to iminoaspartate **44** in preparation for its condensation with dihydroxyacetone phosphate **43**.[95] The A protein catalyzes the condensation to form **45**, cyclization to form **46**, and dehydration to form quinolinate **47**.

The hydroxyl group removed in the second dehydration is not on a carbon β to a carboxyl group, a constellation of atoms contained in the substrates of most other members of the hydro-lyase class. Although the A protein has not been purified to homogeneity, it exhibits features that suggest it contains a Fe-S cluster. *E. coli* quinolinate synthetase is inactivated by O_2 *in vivo* and *in vitro*, but can be reactivated in crude extract incubated anaerobically. The reactivation is prevented by the addition of nonpolar chelators to the crude extract.[96] Protein A contains the motif, C-X_2-C-X_2-C,[97] which is often found in proteins containing Fe-S clusters.[8]

L. Gluconate, Mannonate, Altronate, and Fuconate Dehydratases

D-Gluconate dehydratase [EC 1.2.1.39], D-mannonate dehydratase [EC 4.2.1.8], D-altronate dehydratase [EC 4.2.1.7], and D-fuconate dehydratase [EC 1.2.1.67] function in the hexuronate pathway where they carry out reaction 19 in which the uronic acid, after which they are named **48**, is converted to the corresponding keto acid **49**, a reaction very similar to that catalyzed by dihydroxy-acid dehydratase and phosphogluconate dehydratase.



48 49 (19)

Gluconate dehydratase has been isolated in milligram quantities from *Clostridium pasteurianum*.[98] The enzyme is inactivated by exposure to O_2 and can be reactivated over a period of hours by incubation in solutions containing ferrous ions and thiols, which are common characteristics of Fe-S cluster-containing hydro-lyases. However, there was no mention in the report on the purification of this enzyme of a color associated with it as would be expected if the isolated enzyme contained an intact or partially degraded Fe-S cluster. Furthermore, Mn^{2+}, Co^{2+}, and Mg^{2+} can partially replace the requirement for the Fe^{2+} in the activation reaction, characteristics that are not typical of Fe-S cluster-containing hydro-lyases.

Altronate and mannonate dehydratases have been partially purified from *E. coli*. Altronate dehydratase was found to be unstable in air and required ferrous ions and a thiol for reactivation. Mannonate dehydratase was found to be stable in air in the presence of 0.1 M mercaptoethanol.[99] Subsequent studies have shown that both of these enzymes require a divalent metal for activity with Fe^{2+} or Mn^{2+} in the presence of a thiol providing some degree of activation.[100,101] Maximal reactivation occurred in the presence of both Fe^{2+} and Mn^{2+} along with dithiothreitol; the level of activity reached being 4−7 times that of enzyme reactivated with either metal alone. The presence of S^{2-} did not enhance the reactivation. The reactivated enzymes do not have either optical or EPR spectra characteristic of proteins with Fe-S clusters, and both enzymes contain only one iron per mole of enzyme following reactivation. No activation occurred when Fe^{3+} was substituted for Fe^{2+}.

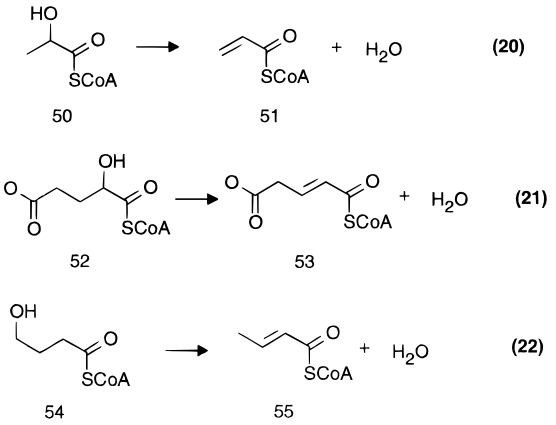
The little information in the literature that exists on fuconate dehydratase suggests that it requires a divalent metal with Fe^{2+} and Co^{2+}, both being effective. One iron per mole is found in the iron-activated enzyme.[102,103]

These results suggest that these four enzymes may be members of a third subclass of hydro-lyases requiring one or two loosely bound, but catalytically essential, divalent metal ions for activity. A cluster of the rubredoxin type would be consistent with the presence of one iron per mole of protein, but no optical spectra typical of a rubredoxin-like iron center have been reported for these proteins. *Pseudomonas* D-(+)-tartrate dehydratase resembles these four enzymes in requiring a divalent cation for activity but lacking a Fe-S cluster.[72,73]

III. Enzymes in the Hydro-lyase Family Containing a Fe-S Cluster That May Perform Both a Catalytic and Redox Function

Several anaerobic bacteria have enzymes with the capacity to dehydrate a number of hydroxyacyl-CoA derivatives. The dehydration of (2*R*)-lactyl-CoA **50** to acrylyl-CoA **51**, (2*R*)-2-hydroxyglutaryl-CoA **52** to (*E*)-glutaconyl-CoA **53**, and 4-hydroxybutyryl-

CoA **54** to crotonyl-CoA **55** shown in reactions 20, 21, and 22, are chemically difficult because the C−H bond to be broken is in the unactivated position β to a carboxyl group. The 2-hydroxyacyl-CoA dehydratases catalyze the removal of water from their substrates by a *syn* elimination.[104,105] An enlightening review of these reactions has been written by Buckel and his colleagues.[106]



(2*R*)-Lactyl-CoA dehydratase functions in the pathway of alanine formation and has been purified from *Clostridium propionicum*.[107] This enzyme requires the addition of titanium(III) citrate, high levels of $MgCl_2$, and substoichiometric amounts of ATP for activity *in vitro*.[104,107] The ATP is hydrolyzed in the reaction mixture and appears to be involved in the activation of the enzyme. The enzyme consists of two O_2 labile proteins, EI and EII. EI is reported to be a single polypeptide with a mass of 27 kDa. An unknown prosthetic group might be required, which could account for the O_2 lability. EII is thought to be an $\alpha_3\beta_3$ hexamer.[104] It contains 0.5 mol of reduced FMN and 0.25 mol of reduced riboflavin per $\alpha\beta$ dimer, and iron and inorganic sulfide in approximately equal amounts.[104,107,108] EPR analysis of EII lead Kuchta *et al.* to propose that the protein contains an unusual [4Fe-4S] cluster and a [3Fe-3/4S] cluster.[104,107] The EPR signal ascribed to the [3Fe-3/4S] cluster is altered by the addition of acrylyl-CoA or lactyl-CoA, suggesting that the binding of substrate to EII alters the environment of the [3Fe-3/4S] cluster.

2-(2*R*)-Hydroxyglutaryl-CoA dehydratase, a key enzyme in the fermentation of glutamate, has been purified from *Acidaminococcus fermentans*.[109,110] This enzyme contains 0.3 mol of reduced riboflavin, 1 mol of reduced FMN, 4 mol of nonheme iron, and 4 mol of sulfide per 100 kDa $\alpha\beta$ heterodimer. Activation of the enzyme requires ATP, $MgCl_2$, Ti(III) citrate,[111] and an activator protein that has been overexpressed and purified from *E. coli*.[110] Analogs of ATP that cannot be hydrolyzed do not support enzyme activation, suggesting that ATP hydrolysis needs to occur during activation. The activator protein, which is extremely sensitive to oxygen, is a 54 kDa homodimer that contains 4 mol of nonheme iron and 3 mol of inorganic sulfide per dimer.

A reversible, transient inactivation of the enzyme system is observed in the presence of the oxidants 2-nitrophenol, 3-nitrophenol, 4-nitrophenol, chloramphenicol, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, and 4-nitrobenzoate (1−10 μM). Irreversible inactivation occurs in the presence of 6 μM nitrite and 30 μM hydroxylamine.[110] The EII component of lactyl-CoA dehydratase and the 2-hydroxglutaryl-CoA dehydratase from *A. fermentans* are similar with respect to the reaction catalyzed, oxygen lability, the presence of [Fe-S] clusters and reduced riboflavin and FMN, and the requirements for activation.

2-(2*R*)-Hydroxyglutaryl-CoA dehydratase has also been isolated from *Fusobacterium nucleatum*.[112] The *F. nucleatum* enzyme is a 112 kDa $\alpha\beta\gamma$ trimer that contains 0.5 mol of reduced riboflavin, 3.5 mol of nonheme iron, and acid-labile sulfide. Titanium(III) citrate, ATP, and $MgCl_2$ are required for activation of the dehydratase, but no additional activator protein is required. This has led to speculation that the γ subunit of this protein is analogous to the activator protein of the *A. fermentans* system and EII of the *C. propionicum* lactyl-CoA dehydratase system. No FMN has been reported to be associated with 2-(2*R*)-hydroxyglutaryl-CoA dehydratase from *F. nucleatum*. It should be noted, however, that the initial studies of the enzyme from *A. fermentans* reported that neither riboflavin nor FMN was associated with the pure enzyme.[109] A dehydratase system similar to that found in *A. fermentans* has also been identified in *Clostridium symbiosum*.[110]

The reversible dehydration of 4-hydroxybutyryl-CoA to crotonyl-CoA is similar to the reactions catalyzed by the 2-hydroxylacyl-CoA dehydratases described above in that it requires the cleavage of an unactivated C−H bond. However, the 4-hydroxybutyryl-CoA dehydratases from *Clostridium aminobutyricum*[113] and *Clostridium kluyveri*[114] have a number of features that distinguish them from the 2-hydroxyl-acylCoA dehydratases. The 4-hydroxybutyryl-CoA dehydratases are homotetramers (~230 kDa) that contain 2 mol of FAD and at least 13 mol of nonheme iron and 10 mol of inorganic sulfide per tetramer, probably in the form of [Fe-S] clusters. The FAD is present in the oxidized state and reduction by sodium dithionite reversibly inactivates the dehydratases. The 4-hydroxybutyryl-CoA dehydratases do not require activation by Ti(III) citrate, ATP, and $MgCl_2$ and are not inactivated by oxidants such as 4-nitrophenol and chloramphenicol, which inactivate the 2-hydroxyacyl-CoA dehydratases described above. Both enzymes are irreversibly inactivated by exposure to air, and both possess vinylacetyl-CoA Δ-isomerase activity. The significance of active 4-hydroxybutyryl-CoA dehydratases containing oxidized FAD compared to the presence of reduced riboflavin in the 2-hydroxyacyl-CoA dehydratases is not understood, but might suggest a redox function for the flavins in the dehydration reactions.

A number of proposals concerning the mechanism of action of these unique dehydratases have been made. It has been proposed that they work by a radical mechanism, but no evidence for this exists.[107,111] Buckel and Keese have recently proposed ketyl intermediates for the 2-hydroxyacyl and 4-hydroxyacyl-CoA dehydratases that could be generated by one-electron reduction or oxidation of the thioester substrates, respectively.[115] The role of the [Fe-S]

Table 2. Rate Constants for the Inactivation of Fe-S Cluster Containing Hydro-lyases With O_2^-

hydro-lyase	k_{inact} (M^{-1} s^{-1}) with superoxide dismutase as a reference	k_{inact} (M^{-1} s^{-1}) with cytochrome *c* as reference
E. coli dihydroxy-acid dehydratase	$(1 \pm 0.3) \times 10^{6}$‡	$(1 \pm 0.3) \times 10^{6}$†
E. coli fumarase A	$(6 \pm 3) \times 10^{6}$†	$(2 \pm 0.6) \times 10^{6}$†
E. coli fumarase B	$(5 \pm 2) \times 10^{6}$‡	$(2 \pm 0.6) \times 10^{6}$†
beef heart aconitase	$(3 \pm 2) \times 10^{6}$†	$(6 \pm 2) \times 10^{6}$†‡

‡ Indicates the value is the average of three determinations. † Indicates the value is the average of two determinations. Errors shown were calculated by making multiple determinations, subtracting the lowest value from the highest value measured, and dividing the difference by two. (Reprinted from ref 76. Copyright 1993 by American Society of Biochemistry and Molecular Biology.)

clusters of these enzymes, and the precise mechanism remains elusive. On the basis of the aconitase paradigm one possibility is that the hydroxyl group of the substrates becomes coordinated to an iron of the [Fe-S] clusters of these enzymes, thereby facilitating the elimination of the hydroxyl group. The [Fe-S] clusters might also be involved in the one-electron reduction of the substrates as recently proposed by Buckel and Keese.[115]

IV. The Effect of O_2^- on Fe-S Cluster Containing Members of the Hydro-lyase Class of Enzymes

It has been known for years that *E. coli* dihydroxy-acid dehydratase is inactivated in cells treated with hyperbaric O_2, but the reason for this inactivation remained elusive.[116,117] The recent finding that this enzyme contained a Fe-S cluster led to the hypothesis that the molecular basis for its inactivation was the oxidation and destruction of its Fe-S cluster by an oxygen species. This idea has been shown to be correct.[118] O_2 will destroy the Fe-S cluster of this enzyme *in vitro*, so it seemed reasonable to conclude that the destruction of the cluster *in vivo* under hyberbaric O_2 was due to the direct effect of O_2. However, this enzyme is also inactivated in *E. coli* growing in the presence of paraquat under ambient levels of O_2. Since both paraquat and hyperbaric O_2 generate excess levels of O_2^- *in vivo*, the possibility that the principal inactivating species in the presence of both paraquat and hyperbaric O_2 might be O_2^- was raised and some evidence for this was acquired.[119,120]

Subsequently, an investigation was made into the rates of inactivation by O_2^- of four purified hydro-lyases containing [4Fe-4S] clusters: *E. coli* dihydroxy-acid dehydratase, FumA, FumB, and mammalian aconitase. These enzymes were found to be rapidly inactivated by O_2^- due to the oxidation and ensuing destruction of their Fe-S clusters.[76] The values for the second-order rate constants for inactivation of these enzymes by O_2^- are shown in Table 2.

An independent estimate for the second-order rate constant for the inactivation of *E. coli* aconitase by O_2^- has been made by the Fridovich laboratory, and the value reported, 2×10^9 M^{-1} s^{-1}(at the diffusion control limit), is much higher than the values given in Table 2.[121] This higher value was based on experiments performed in *E. coli* crude extract, an experimental situation fraught with difficulties. It has subsequently been corrected by the group that originally made it; and in its place a 100-fold lower value, 3×10^7 M^{-1} s^{-1}, has been reported.[122]

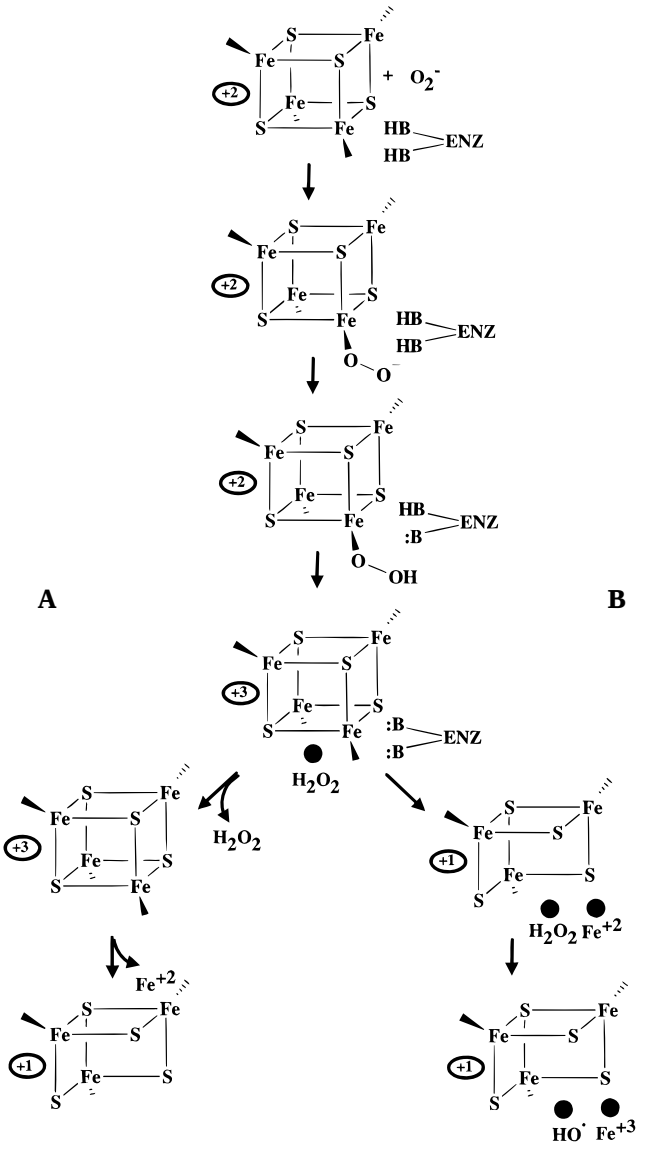
A second-order rate constant for the reaction of O_2^- with *E. coli* 6-phosphogluconate dehydratase 2×10^8 M^{-1} s^{-1}, has also been reported from experiments performed in *E. coli* crude extract.[84] This value is approximately 100-fold higher than the values given in Table 1 for *E. coli* dihydroxy-acid dehydratase, FumA, FumB, and mammalian aconitase. In view of the difficulties in obtaining a reliable value in crude extract,[121] this rate constant may also be an overestimate.

Since it is uncommon for enzymes, even those with Fe-S clusters, to be inactivated by O_2^- (e.g., xanthine oxidase, an enzyme commonly used to generate O_2^- contains a Fe-S cluster, but it is not inactivated by O_2^-), these results raise the question: why are these hydro-lyases so susceptible to inactivation by O_2^-. There are four factors that could contribute: (1) unlike the Fe-S clusters in other proteins, O_2^- probably has direct access to the Fe-S clusters of the hydro-lyases (recall that the X-ray crystal structure of mammalian mitochndrial aconitase shows that one iron of the cluster is exposed to solvent, and this may be true with clusters of *E. coli* dihydroxy-acid dehydratase, FumA, and FumB, 6-phosphogluconate dehydratase and cytoplasmic aconitase as well), (2) the active sites of each of these enzymes normally bind anionic substrates and may facilitate binding of O_2^-, (3) the oxidation of the clusters of the hydro-lyases by O_2^- is thermodynamically favorable, (4) the oxidation of the clusters of these hydro-lyases by O_2^- may be assisted kinetically by enzymic residues that normally participate in acid/base chemistry during catalysis.

With regard to points three and four, the redox potential for the reduction of O_2^- shows that it should be a capable oxidant for a Fe-S cluster. However, oxidation by O_2^- is slow in the unprotonated form, but much faster in the protonated form. Since the protonated form is a minor species at physiological pH ($pK_a = 4.69$), O_2^- is kinetically rather inept as an oxidant in biological milieu.[123−125] However, once O_2^- is bound to the Fe-S clusters of the hydro-lyases, it could be protonated by an acidic group in the active site. HO_2 bound to the exposed iron of the cluster would be able to express its full potency as an oxidant. A diagram of the proposed events involved in the inactivation of Fe-S cluster containing hydro-lyases by O_2^- is shown in Scheme 7 (the large black dots in the Scheme indicate a physical association of the chemical species below the dots with the enzyme active site).

Apparently the events outlined in the right-hand fork of Scheme 7 that lead to the formation of hydroxyl radical followed by the irreversible inactivation of the enzyme either rarely occur, or if a hydroxyl radical is formed it does not react with the protein since irreversible inactivation does not take place in the majority of the inactivation events. For example, it has been shown that after the Fe-S cluster of dihydroxy-acid dehydratase is removed by hyperbaric

Scheme 7. (A) Reversible Inactivation Due to Cluster Degradation and (B) Irreversible Inactivation Due to Oxidation of Active Site Residue[a]



[a] In A, the H_2O_2 leaves the active site before Fe^{+2} is released from the cluster, and in B, H_2O_2 remains in the active site until Fe^{+2} is released from the cluster. Hydroxyl radical is formed in active site.

O_2 *in vivo* (apparently by O_2^-) the apoprotein of this enzyme remains in a form that can be reactivated. When the hyperbaric O_2 stress is relieved, the cluster is remade on the apoprotein and the enzymatic activity recovers.[124]

Since the Fe-S clusters of the hydro-lyases are so susceptible to damage by O_2^-, it seems possible one important role of superoxide dismutase is to protect the Fe-S clusters of hydro-lyases from destruction. This possibility was tested by measuring the levels of 6-phosphogluconate dehydratase activity in two *E. coli* strains, one wild-type and one lacking superoxide dismutase, that were grown in the presence of ambient O_2. The activity of 6-phosphogluconate dehydratase was much lower in the strain lacking superoxide dismutase.[84] Similar results have been obtained with dihydroxy-acid dehydratase[126] and aconitase.[127,128]

In combination, these studies suggest that a dynamic equilibrium may exist between the apo and holo forms of these hydro-lyases that is driven in one direction by O_2- and O_2^--mediated cluster oxidation and destruction and in the other direction by cluster resynthesis. The fraction of a hydro-lyase in each state would depend on the rates of cluster breakdown and resynthesis. This may mean that these enzymes cycle between the apo- and holoprotein several times during their lifetime. Directly measuring the flux in this dynamic equilibrium under "normal" growth conditions is difficult, but has been attempted in the case of aconitase.[127,128]

The molecular events that contribute to the toxicity of O_2^- are poorly understood, but the toxicity seems incontrovertible because superoxide dismutase null mutants are invariably seriously impaired when grown under aerobic conditions. Among other things, the lack of superoxide dismutase *in vivo* leads to DNA damage. How O_2^- damages DNA has not been clear. Traditionally, it has been thought that O_2^- promotes Fenton chemistry by reducing Fe^{3+} to Fe^{2+}, which in turn would reduce endogenous hydrogen peroxide to generate the hydroxyl radical that could damage DNA.[129] However, a recent study has shown that O_2^- may not act as a reductant of Fe^{3+} *in vivo*.[130]

This study suggests that a primary toxic act of O_2^- may be to destroy the Fe-S clusters of the hydro-lyase class of enzymes. The one Fe^{2+} and three Fe^{3+} released from these clusters on complete destruction would be set free in the cell. The Fe^{3+} could be reduced by cellular reductants such as glutathione to Fe^{2+}. Some of the Fe^{2+} could become associated with DNA and in consort with hydrogen peroxide could generate hydroxyl radicals and damage the DNA.[130] The results of this study shift the emphasis of the role of O_2^- from direct involvement in Fenton chemistry as a Fe^{3+} reductant, to the indirect role of releasing iron from the Fe-S clusters of members of the hydro-lyase class. The freed iron then may participate in Fenton chemistry upon reduction (by molecules other than O_2^-).

Compared to the labile [4Fe-4S] clusters found in most hydro-lyases, the [2Fe-2S] cluster of spinach dihydroxy-acid dehydratase is unique in not being affected by either O_2 or O_2^-. Why? Two possibilities are discussed.

First, spinach dihydroxy-acid dehydratase may be stable to O_2^- and O_2 because the protein protects the cluster. An intriguing possibility is that the catalytically active iron in this enzyme's Fe-S cluster has two ligands from protein in the resting state and, for this reason, is not directly accessible to O_2 and O_2^-. If this is the case, one ligand may need to be removed during catalysis in order for the hydroxyl group of the substrate to become a ligand to the cluster. This could require the enzyme to undergo a conformational change as the substrate binds and may as a result slow the enzymatic rate. In this regard, it is worth noting that the k_{cat}/K_m for spinach dihydroxy-acid dehydratase (33 M^{-1} s^{-1})[79] is 1 order of magnitude lower than the k_{cat}/K_m of *E. coli* dihydroxy-acid dehydratase(530 M^{-1} s^{-1}).[75] Could it be that some of the potential catalytic power of the spinach dihydroxy-acid dehydratase has been sacrificed for in-

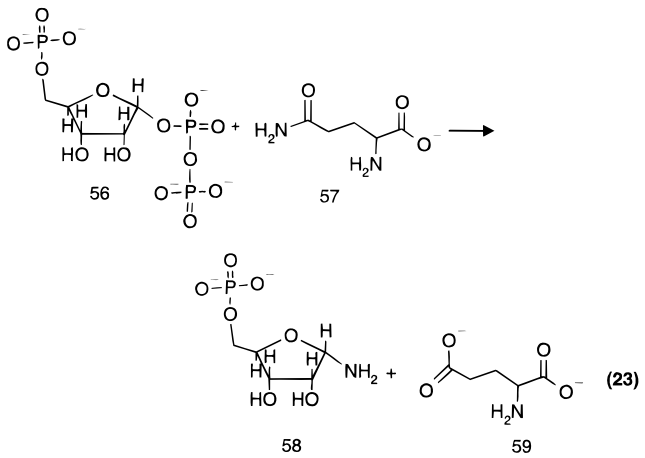
creased stability to O_2 and O_2^-? The resistance of spinach enzyme to oxygen species may be of physiological significance because the enzyme resides in the chloroplast, an organelle with unusually high concentrations of both O_2 and O_2^-.

The second possibility is that the stability of spinach dihydroxy-acid dehydratase may be inherent in the [2Fe-2S] itself. Perhaps the cluster of spinach dihydroxy-acid dehydratase is resistant to oxidation because both of the iron atoms in the spinach cluster are in the ferric oxidation state, whereas two of the irons are in the ferrous state in $[4Fe\text{-}4S]^{2+}$ clusters. Oxidation of the cluster of the spinach enzyme would only be possible by withdrawing electrons from the sulfides. If the intrinsic properties of its [2Fe-2S] cluster is the main factor contributing to the stability of the spinach enzyme in the presence of O_2 and O_2^- (as opposed to the protein itself protecting the cluster), it leads to the prediction that plant isopropylmalate isomerase, another hydro-lyase residing in chloroplasts, will also be found to contain a [2Fe-2S] cluster and be stable to both O_2 and O_2^- (as discussed previously, yeast isopropylmalate isomerase contains a [4Fe-4S] cluster that is unstable to O_2 and O_2^-).

V. Enzymes Not in the Hydro-lyase Class in Which the Fe-S Clusters Appear To Perform a Structural and/or Regulatory Function

A. Glutamine 5-Phosphoribosyl-1-pyrophosphate Amidotransferase

This enzyme [EC 2.4.2.14] catalyzes the transfer of an amino group from glutamine **57** to ribose-5-phosphate **56** to form **58**, the first committed precursor in the purine biosynthetic pathway, as shown in reaction 23. Two excellent reviews that address this enzyme have been published by leaders in the field.[131,132] The enzyme has been isolated from *Bacillus subtilis*,[133] *E. coli*,[134] avian liver,[135,136] and several mammalian sources including human placenta.[137]



The enzyme purified from *B. subtilis* contains iron and sulfide. This enzyme rapidly loses activity in the presence of O_2 and has to be isolated under anaerobic conditions to obtain the native form. This enzyme has a Mössbauer spectrum characteristic of $[4Fe\text{-}4S]^{2+,+}$ clusters (single symmetrical quadrupole doublet; isomer shift = 0.44 mm/s; quadrupole splitting value = 1.19 mm/s).[131,138] A good EPR spectrum of the reduced $[4Fe\text{-}4S]^+$ cluster containing form of this enzyme has not been obtained because of its very low redox potential (< -620 mV)[139] and the fact that the majority of the reduced cluster exists in a $S = {}^3/_2$ spin state.[140] A good EPR spectrum of the $[3Fe\text{-}4S]^+$ cluster-containing form of this enzyme has not been obtained because this species is unstable. The recently solved X-ray crystal structure of this enzyme clearly demonstrates the presence of a [4Fe-4S] cluster.[141]

The X-ray crystal structure of the *B. subtilis* enzyme shows that in contrast to aconitase, all four iron atoms have ligands from the protein provided by Cys_{236}, Cys_{382}, Cys_{437}, and $Cys_{440,141}$. The differences between the ligation state of aconitase and the amidotransferase are manifest in their Mössbauer spectra, which clearly show the iron atoms in the cluster of glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase are in similar environments, whereas the iron atoms in aconitase exist in two environments. This enzyme contains the motif $Cys_{434}\text{-}X_2\text{-}Cys_{437}\text{-}X_2\text{-}Cys_{440}$, and it was expected that these three cysteines would each provide protein ligands for the cluster. The X-ray structure proved that was not the case. This example demonstrates that caution should be used in concluding that cysteines that appear in such motifs all provide ligands to the protein's Fe-S cluster.

The amidotransferases from avian and mammalian sources also appear to contain a [4Fe-4S] cluster since they (1) have 3−4 atoms of iron/subunit of enzyme, (2) have a brown color due to absorbance with a peak in the 410−420 nm region, and (3) are inactivated by metal chelators, O_2, and O_2^-.[135−137] The amino acid sequences of *B. subtilis*, avian, and human glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase share significant sequence homology. Five cysteine residue are conserved among these enzymes; four of them correspond to the aforementioned cysteines in the *B. subtilis* enzyme that provide ligands to the cluster. (The fifth conserved cysteine is found in the N-terminal portion of the protein where it performs a critical function as the nucleophile in the glutaminase activity of this enzyme.)

In contrast, the amidotransferase from *E. coli* does not contain iron and sulfide, is not colored, is not inactivated by chelators, or O_2, and the four cysteines furnishing ligands to the Fe-S cluster that are conserved among the enzymes described in the preceding paragraph are not conserved in the sequence of *E. coli* glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase.[132] Clearly the *E. coli* enzyme does not contain a Fe-S cluster. Since the amino acid sequences of the yeast, *Neurospora*, and *H. influenzae* glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferases closely resemble the *E. coli* amidotransferase, it is unlikely that they will be found to contain Fe-S clusters.[132]

What could the role of the Fe-S cluster in the *B. subtilis*, avian, and mammalian glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase be? Its presence is essential for enzyme activity, but the cluster does not perform a direct function in cataly-

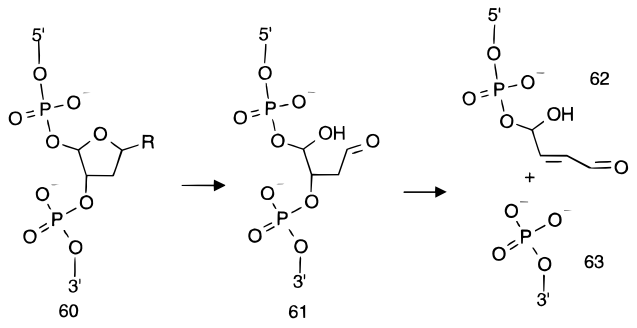
sis.[131] The cluster appears to perform a structural function in these enzymes that helps orient the groups in the active site appropriately for catalysis. It also appears to perform a regulatory function in *B. subtilis* as described below.

When *B. subtilis* cells are growing in minimal media, functional amidotransferase is essential for purine synthesis. The O_2 tension *in vivo* during logarithmic growth is low because O_2 is consumed by respiration. Under these conditions, the amidotransferase is active. When a nutrient becomes limiting and growth slows, the internal concentration of O_2 rises, and the need for the *de novo* synthesis of purines declines. The increased O_2 tends to oxidize the Fe-S cluster of the amidotransferase, inactivate the enzyme, and thereby decrease the flux in the purine biosynthetic pathway. The loss of cluster from the enzyme leads to proteolysis because it changes the structure of the protein to the point that it becomes susceptible to intracellular proteases and it is degraded.[133,142,143] Thus the relation between the rate of purine synthesis and growth appears to be mediated by the effect of O_2 on the Fe-S cluster of this enzyme.

If the regulatory role for the amidotransferase Fe-S cluster described in the preceding paragraph is correct, what are we to make of the fact that the avian and mammalian amidotransferases also have oxygen sensitive Fe-S clusters? The cluster in these enzymes appears to perform a structural role. Does it also perform a regulatory role in purine biosynthesis, or a different regulatory role? If not, what regulates these enzymes, and why has a cluster free enzyme not evolved in these species?

B. Endonuclease III

Endonuclease III from *E. coli* is a DNA repair enzyme that exhibits both DNA *N*-glycosylase and apurinic/apyrimidinic lyase activities.[144−146] The DNA *N*-glycosylase activity removes pyrimidines damaged by ring saturation, contraction, or fragmentation.[147] The lyase activity of the enzyme introduces a single-strand break in the DNA at the apyrimidinic site via a β-elimination reaction[100] diagrammed in reaction 24.[148,149]



R = hydroxyl, urea, or modified pyrimidine or purine base

(24)

E. coli endonuclease III is encoded by the *n*th gene, which has been cloned and sequenced. The protein has been overexpressed, purified, and found to be a monomer.[150] As might be expected of a protein that binds DNA, endonuclease III is basic with a p*I* of ~10.

Endonuclease III contains 3−4 iron and sulfide atoms per monomer.[151] Oxidation of this enzyme with ferricyanide produces a species that exhibits an EPR signal near $g_{av} = 2.01$, characteristic of [3Fe-4S] clusters. The protein has a Mössbauer spectrum characteristic of $[4Fe\text{-}4S]^{2+,+}$ clusters (single symmetrical quadrupole doublet; isomer shift = 0.44 mm/s; quadrupole splitting value = 1.18 mm/s) that is very similar to the spectrum of glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase. These data clearly demonstrate that the native enzyme contains a $[4Fe\text{-}4S]^{2+,+}$ cluster and that all of the iron atoms in the cluster are in a similar environment. Endonuclease III is the first documented Fe-S cluster containing DNA repair enzyme. The inability to reduce the $[4Fe\text{-}4S]^{2+}$ cluster with sodium dithionite (at pH 10) or by deazaflavin-mediated photochemical reduction suggests a midpoint potential of <-600 mV for the $[4Fe\text{-}4S]^{2+,+}$.[152] This makes it unlikely that the cluster of endonuclease III is reduced *in vivo*. The [4Fe-4S] cluster of the native enzyme is resistant to oxidation by O_2. Apo-endonuclease III is inactive and tends to precipitate from solution, but can be partially reactivated *in vitro* in the presence of iron and sulfide.[153]

The X-ray crystal structure of endonuclease III shows that the four iron atoms of the cluster are ligated by the cysteine residues in the following motif: $Cys_{187}\text{-}X_6\text{-}Cys_{194}\text{-}X_2\text{-}Cys_{197}\text{-}X_5\text{-}Cys_{203}$, which is found in a DNA binding loop at the C-terminal end of this protein.[154] Both the spacing of the cysteine residues and the unusually short sequence-continuous region containing them are distinct among [4Fe-4S] cluster-containing proteins of known structure. In contrast to the Fe-S cluster of aconitase and similar to the cluster in glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase, this protein provides a sulfur ligand to each iron atom in the Fe-S cluster. No changes in the resonance Raman or Mössbauer spectra are observed upon substrate or inhibitor binding to endonuclease III, suggesting that substrate does not interact with the Fe-S cluster.[151,152] All available evidence suggests that the Fe-S cluster of endonuclease III does not directly participate in catalysis.

On the basis of the analysis of the crystal structure of the enzyme, Kuo *et al.* have proposed that the Fe-S cluster is involved in aligning conserved positively charged residues for interaction with the DNA.[154] Mutating of one of these residues to a negatively charged residue results in a mutant enzyme with a >100-fold K_m and an essentially unchanged k_{cat}.[155] These results demonstrate that the mutation markedly affects DNA binding and are consistent with the hypothesis that these conserved charged residues interact with DNA.

On the basis of a refined crystal structure of endonuclease III and sequence comparisons, Thayer *et al.* have recently proposed that the DNA binding loop containing the Fe-S cluster of endonuclease III might be a common structural element of DNA binding proteins.[155] The motif $Cys\text{-}X_6\text{-}Cys\text{-}X_2\text{-}Cys\text{-}X_5\text{-}Cys$ is conserved in a number of DNA binding proteins including (1) the MutY protein of *E. coli*,[156,157] (2) a protein from *Salmonella typhimurium*

homologous to MutY,[158] (3) a putative thymine-DNA glycosylase that has been identified in the archaebacterium *Methanobacterium thermoformicicum*,[159] (4) the recently purified and cloned ultraviolet endonuclease from *Micrococcus luteus*,[160] and (5) a homolog of endonuclease III that has recently been purified from calf thymus.[161] Analysis of translated amino acid sequences suggests that *Caenorhabditis elegans*, *Homo sapiens*, and *Rattus* sp. also have endonuclease III-like proteins that contain this novel motif.[161] A family of DNA repair enzymes that shares a common mode of DNA binding appears to be present throughout phylogeny. It should also be noted that the activity related T4 bacteriophage endonuclease V lacks a Fe-S cluster.[162]

VI. Regulatory Proteins without Known Enzymatic Activity in Which the Fe-S Cluster Appears To Be Involved in Sensing O_2 or O_2^-

Recently the proteins encoded by the *soxR* and *fnr* genes have been found to contain Fe-S clusters. SoxR and FNR have no known enzymatic activity, their sole function appears to be regulation, and their Fe-S clusters may eventually be found to undergo some kind of redox chemistry—all reasons to exclude them from this review. However, they are included because the discovery that cytoplasmic aconitase and IRE-BP are the same protein has established that nature knows how to use a Fe-S cluster in a protein for the dual functions of catalysis and regulation. It seems possible that additional examples of this phenomenon will be found among the proteins considered in this review. On that basis, a brief review of SoxR and FNR seems justified because any understanding of how Fe-S clusters participate in regulation could eventually be pertinent to the enzymes considered in this review.

A. SoxR

SoxR and SoxS in tandem control the expression of the *soxRS* regulon in *E. coli* and thereby control the expression of several genes whose products are made in response to O_2^-. SoxR responds to O_2^- by either sensing it directly or indirectly and it in turn governs the expression of SoxS, which is a transcriptional regulator that governs the expression of the dozen or so genes in the regulon.

SoxR is a homodimer, contains two irons per monomer when it is carefully isolated, has an optical spectrum characteristic of proteins with [2Fe-2S] clusters, and upon reduction exhibits a $S = {}^1/_2$ axial EPR spectrum (*g* values 2.01, 1.92, 1.90). All of these features are characteristic of a protein with a $[2Fe\text{-}2S]^{2+,+}$ cluster.[163,164] The cluster can be removed and reconstituted *in vitro* in the presence of iron, sulfide, and mercaptoethanol or in the presence of iron, cysteine, NifS, and dithiothreitol.[165] Sox R contains only four cysteines that are grouped in the following stretch of 12 amino acids near its C-terminal end: $C\text{-}X_2\text{-}C\text{-}X\text{-}C\text{-}X_5\text{-}C$. These cysteines appear to be part of a center that responds to O_2^-.[166−169] Since these are the only four cysteines in the protein, at least some, if not all, of them are likely to be involved in ligating the [2Fe-2S] cluster.

Although the Fe-S cluster of this enzyme appears to be involved in sensing the presence of O_2^-, it is not known how this occurs. The simple model of the cluster being present *in vivo* in the absence of O_2^-, and then removed in the presence of O_2^- to form the apoprotein (as in the case of the hydro-lyase *E. coli* dihydroxy-acid dehydratase) is not consistent with the results to date.[170] Further studies of this protein are eagerly awaited.

B. FNR

The *E. coli* transcription factor FNR controls the expression of proteins needed under anaerobic conditions. In the absence of O_2, FNR is converted to an active form that up-regulates the expression of several proteins by binding directly to DNA. The FNR protein consists of two domains, the N-terminus that is thought to be responsible for sensing O_2 and a DNA binding domain that constitutes the rest of the protein. The protein contains only five cysteines, four of which are grouped in the O_2-sensing domain in the following stretch of 14 amino acids: $C\text{-}X_3\text{-}C\text{-}X_2\text{-}C\text{-}X_5\text{-}C$.[171] The FNR protein has been difficult to isolate in its active DNA-binding state because of the lability of this activity to O_2. Prior work had pointed to a role for iron in this protein in sensing O_2, but the exact role remained unclear.[172−174]

E. coli strains containing mutant FNR proteins with more stable DNA-binding activity have recently been isolated. When isolated in their active DNA-binding form these proteins are brown in color, have optical spectra typical of Fe-S cluster containing proteins, contain a significant amount of iron and sulfide, and exhibit EPR spectra typical of proteins with $[3Fe\text{-}4S]^+$ clusters. These results strongly suggests that wild-type FNR in cells grown anaerobically contains a Fe-S cluster. It is highly likely that at least some of the cysteines in the N-terminal O_2 sensing domain are ligands to the cluster.

When the Fe-S cluster is removed from the mutant FNR proteins with an iron chelator, their DNA-binding affinity is greatly reduced. When the Fe-S cluster is rebuilt on the apoprotein *in vitro*, the high DNA-binding affinity returns.[175] This change in DNA-binding affinity appears to be due to an effect of the presence of a Fe-S cluster on the multimeric state of the protein. The dimeric DNA-binding form of the protein is stable when the Fe-S cluster is present; however, when the cluster is lost, the protein dissociates into monomers, which have low DNA-binding affinity.[176] Taken together these results suggest that this protein senses O_2 through its [4Fe-4S] cluster with the effect of the O_2 being to remove the cluster.

VII. Summary and Unanswered Questions

The Fe-S clusters of the proteins discussed in this review have been shown to perform functions in (1) catalysis, (2) control of protein structure, and (3) regulation. The majority of these Fe-S clusters are only known to perform one of these functions. However, the recent discovery that the Fe-S cluster of cytosolic aconitase/IRE-BP performs two and possibly all three of these functions, and that the Fe-S cluster

of *B. subtilis* glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase performs two of them, brings up the possibility that the Fe-S clusters of other proteins discussed in this review may have additional unknown functions. In the Introduction, we referred to the line of thought that members of the Fe-S cluster-containing subclass of hydro-lyases may be evolutionary relics.[11−13] While this may be correct in those cases where Fe-S clusters are used by Nature to accomplish only a catalytic function (as demonstrated by clusterless proteins catalyzing the same reaction), in those cases where the proteins in this review are required to play multiple roles, a Fe-S cluster may be the only prosthetic group available that could assist the proteins in playing these roles. An important issue concerning the proteins discussed in this review is which ones use Fe-S clusters to play multiple roles.

While only further work will fully address this issue, consider for the moment the perplexing situation presented by the three fumarase genes in *E. coli*. FumA is expressed under aerobic conditions when an ample iron supply is present; FumB is expressed under anaerobic conditions and is controlled by the anaerobic transcriptional activator FNR; and FumC is controlled by the *soxRS* regulon and is expressed primarily under conditions of oxidative stress or limiting iron.[177−179]

The direction of the fumarase reaction *in vivo* is toward malate during aerobic growth when FumA is expressed and toward fumarate during anaerobic growth when FumB is expressed. One might expect from this that FumA and FumB would be particularly well suited to catalyze the fumarase reaction in the malate direction and fumarate directions, respectively. However, there is nothing in the kinetic properties presently known for these enzymes that fulfills this expectation.

Considering the O_2^- sensitivity of FumA and FumB, it makes sense for FumC to be induced when O_2^- levels are high because FumC is O_2^- resistant. However, it is not clear why it is advantageous to *E. coli* to make either FumA or B instead of FumC when O_2^- levels are low. FumA and FumB do not seem to be more efficient catalysts than FumC.

If the authors were invited to design an *E. coli* cell based on the biochemistry we presently know, our choice of fumarase genes would be *fumC* only. Since *E. coli* has not chosen to do that, either it has been whimsical in its choice of genes for this enzyme, or we are ignorant of a significant part of its biochemistry. We think the latter possibility has the higher probability. This leads directly to the possibility that the Fe-S clusters in FumA and FumB may have a role in addition to catalysis. The recent finding that the Fe-S cluster of cytoplasmic aconitase/IRE-BP has a regulatory role in mammals along with its catalytic role[34] seems to strengthen this possibility.

The question arises again with glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase because metal-free and Fe-S cluster-containing forms of this enzyme are known. A role for the Fe-S cluster of the *B. subtilis* enzyme has been proposed, but it does not seem to be applicable to the avian and mammalian enzymes. Do these Fe-S cluster containing enzymes perform functions in addition to catalysis that could not be performed by the metal-free enzyme? It is hoped that additional work on these fascinating enzymes will help us understand why in some cases nature has chosen enzymes with Fe-S clusters to catalyze certain reactions and in other cases has chosen metal-free enzymes to catalyze the same reaction.

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