Advances in Inorganic Biochemistry 4

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ADVANCES IN INORGANIC BIOCHEMISTRY

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PREFACE

The last two volumes of this series have had specific titles in addition to the series title. Thus Volume 2, which was edited by D. W. Darnall and R. G. Wilkins, was entitled "Methods for Determining Metal Ion Environments in Proteins," and Volume 3 was called "Metal Ions in Genetic Information Transfer." Those volumes constituted deliberate attempts to bring together topics with a narrower focus than that suggested by the series title.

Nevertheless, it remains a major purpose of Advances in Inorganic Biochemistry, in line with other Advances series, to bring the reader up-to-date on a variety of topics that are held together only by the wider focus of the series as a whole. Volume 1 dealt with a range of subjects, and Volume 4 resembles Volume 1 in this respect. It is essentially an unstructured book, although we have attempted to put next to each other chapters with some common features. The first three chapters all involve iron, Chapters 4 and 5 are on cleavage enzymes, Chapter 6 discusses lanthanide probes that can be used as calcium analogs while while Chapter 7 is on calcium ATPase, and Chapter 8, like Chapters 5 and 6, makes extensive use of applications of magnetic resonance techniques.

Chapters 1, 2, 4, and 5 update material contained in the treatise on Inorganic Biochemistry, published in 1973. Chapters 3, 6, 7, and 8 contain subjects that have become of interest since that time.

Volume 5 in the series will be co-edited by Elizabeth Theil, and will be entitled "Iron Binding Proteins Without Cofactors or Sulfur Clusters." Volume 6 will again be unstructured.

Gunther L. Eichhorn
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May, 1982

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CONTENTS

1

BIOINORGANIC CHEMISTRY OF NITROGENASE

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I. INTRODUCTION

Nitrogenase presents some of the most complicated and interesting problems to be solved in biochemistry. We have narrowed the scope of this review to the physical and chemical characteristics of this two-component protein system that acts as a catalyst for the reduction of dinitrogen to ammonia. We also include a brief discussion of certain metal-sulfur compounds thought to reproduce some of the structural properties of the protein prosthetic groups, as well as a brief study of the chemistry of low-valent molybdenum compounds which may be relevant to nitrogen fixation. Other recent reviews written from other various points of view are found in (1,2,3,4).

The ability to fix nitrogen is possessed by a variety of prokaryotes, of which the following is but a partial list: anaerobic (Clostridium pasteurianum, Desulfovibrio vulgaris, Desulfatomaculum ruminis); facultatively anaerobic (Klebsiella pneumoniae, Bacillus polymyxa); aerobic (Azotobacter vinelandii); photosynthetic (Rhodospirillum rubrum; and symbiotic (Rhizobium japonicum) bacteria all are represented. Several varieties of blue-green algae, including Anabaena cylindrica and Gloeocapsa alpicola, also fix nitrogen. The nitrogenase proteins are extremely oxygen sensitive; thus, it is of interest to know how they are protected from oxygen damage in aerobes. Some nitrogen-fixing organisms exist either as free-living soil bacteria or symbiotically in root nodules and in some circumstances provide a large fraction of the agricultural requirement of fixed nitrogen. The possibilities of increasing that fraction have given impetus to the study of nitrogen-fixing systems. Genetic modification to enable agriculturally important plants to fix nitrogen, extension of the symbiosis to other crop plants, or design of a low temperature and pressure process for industrial production of ammonia from atmospheric dinitrogen would be obvious boons. These goals might be achieved with knowledge derived from studies of the biochemistry of biological nitrogen fixation.

The nitrogenase protein system is composed of two proteins, the iron protein and the molybdenum-iron protein, situated at the end of an electron transport chain. The iron protein accepts electrons from this chain and acts as the

specific electron donor to the molybdenum-iron protein. No substitute, biological or synthetic, has been found for the iron protein in this role. The molybdenum-iron protein presumably uses these electrons for the reduction of dinitrogen and a variety of abiological substrates. A third nitrogenase protein that acts in a regulatory fashion upon the iron protein has been isolated from *Rhodospirillum rubrum* (5,6).

Workers interested in the catalytic aspects of nitrogen fixation should be aware that *nif*, the collection of genes that confer the ability to reduce dinitrogen, specifies a vary large set of proteins. About seventeen genes (in a single segment of DNA) comprising seven operons are required for the functioning system (see, for example (7)). Only three of these genes specify the peptides of the iron protein and the molybdenum-iron protein. The remainder have to do with regulation of nitrogenase (in response to ammonia, metals, and oxygen), accumulation of molybdenum and iron, processing of proteins and cofactors, and electron transport. These latter genes, serving to create the conditions for the manufacture and operation of the nitrogen-reducing enzyme in the cell, provide an enormous field of inquiry that is just beginning to be explored.

Among the important chemical questions about the enzyme nitrogenase yet to be answered are 1) Why is the iron protein/molybdenum-iron protein interaction so specific? 2) How, if at all, are electrons stored and moved about within the molybdenum-iron protein? 3) What is the nature of the prosthetic group at which substrate reduction occurs? and 4) What is the mechanism of the reduction of substrates? We will attempt to give the evidence pertaining to the answers to these questions in the following sections, but the reader will perceive much further work to be done before a unified picture of the mechanism of this system appears.

II. THE IRON PROTEIN

A. Protein Structure

The iron protein of nitrogenase may accept electrons from either a ferredoxin or flavodoxin *in vivo* or from sodium dithionite *in vitro*. In the presence of MgATP the reduced iron protein is able to transfer electrons to the molybdenum-iron protein, leading eventually to substrate reduction.

The protein from a variety of species is a dimer of identical subunits with a species-dependent total molecular weight of 57,000 to 73,000. Identity of the subunits is suggested both by sedimentation equilibrium experiments and by polyacrylamide disc electrophoresis in the presence of sodium dodecylsulfate and either 2-mercaptoethanol or sodium mersalyl (8). The protein is extremely

oxygen-sensitive, accounting for the difficulty in isolation of highly active purified protein as well as for the lack of success so far in attempts at its crystallization.

The complete amino acid sequences of the iron protein subunits of Clostridium pasteurianum (9) and Klebsiella pneumoniae (10) and the partial sequence of the protein from Azotobacter vinelandii (11) have been determined. Certain regions of the sequences are highly conserved (90-95% homology among the three species); these regions include five cysteine residues per monomer. The persistence of these regions is important since cysteine residues are generally the site of attachment of iron-sulfur clusters in ferredoxins.

B. Characterization of the Iron-Sulfur Cluster

*The protein has been determined to contain four iron atoms and four acid-*labile sulfur atoms per dimer (8). An Fe_4S_4 cluster may be extruded from the iron protein and identified either by reconstitution into the apo-protein of a known four-iron ferredoxin or by comparison of optical spectra with those of synthetic Fe_4S_4 clusters (12,13). This single cluster appears to account for all of the iron in the protein. The identification of a single cluster in a dimeric protein suggests that either the protein is asymmetric (with the cluster wholly contained within one subunit) or that the cluster bridges the two subunits. Since only four cysteines are required to bind the cluster, six cysteines (per dimer) conserved in the sequences of three species are left to play an unknown, potentially important role.

The iron protein may be oxidized irreversibly by oxygen, or reversibly by a variety of organic and inorganic reagents. The optical spectrum of the reduced protein is featureless, while that of the reversibly oxidized protein has a broad shoulder near 420 to 450 nm (8). At low temperatures (<30 K) the reduced protein exhibits a rhombic EPR signal, resembling that of reduced spinach ferredoxin, with apparent g values of 2.25, 1.94, and 1.84 (8,14). The reversibly *oxidized protein is EPR-silent. Observation of the EPR signal height during ox-*idative titration (with ferricyanide) or reductive titration (with dithionite) allowed determination of a reduction potential of -294 ± 20 mV (NHE) (15). Preparation of irreversibly oxidized protein by exposure to oxygen or to an excess of ferricyanide leads to protein which has no enzymatic activity and which exhibits an EPR signal like that of a radical species.

Integration of the EPR signal of highly purified, reduced iron protein yields a puzzling result. The protein from Clostridium shows 0.2 spins per molecule (12,16,17), that from Klebsiella 0.45 spins per molecule (18), and that from

Azotobacter 0.24 spins per molecule (19). Normally the paramagnetic states of simple iron-sulfur proteins yields EPR spectra which integrate easily (at sufficiently low temperatures) to give one spin per protein molecule. The extreme oxygen-lability of this protein, as well as the variability in specific activity reported for different preparations from the same organism, would suggest that this unexpected result arises from contamination by proteins lacking functional clusters (20). Evidence against this suggestion comes from studies of the reactivity of the iron atoms in the cluster (21,22).

Chelating agents with a high affinity for Fe^{2+} have been used to probe the accessibility of the cluster in the iron protein (23). Addition of α,α-dipyridyl or bathophenanthroline disulfonate to purified iron protein leads to complexation of a small amount of iron. Addition of MgATP to a solution of protein and chelating agent results in complexation of virtually all of the iron in the protein within a few minutes, suggesting that the cluster becomes more exposed to the solvent upon binding of MgATP to the protein. Oxidative inactivation of the protein similarly enhances the chelation of cluster iron. In that case the iron apparently is rendered more reactive either by the increased accessibility of the cluster or by its destruction. The amount of iron chelated in the absence of MgATP is thought to represent protein that is oxygen-inactivated; thus, the additional iron chelated upon introduction of the nucleotide salt represents the number of active, protein-bound clusters. Using data from these experiments, the specific activity in an acetylene reduction assay of pure iron protein was calculated to be 3.1 μmol ethylene produced $min^{-1}mg^{-1}$.

This iron chelation phenomenon was used to estimate the difference in extinction coefficient between the reduced and oxidized forms of protein containing intact iron-sulfur clusters. Use of the value obtained, 6600 $M^{-1}cm^{-1}$ at 430 nm, permitted measurement of the number of electrons transferred per molecule of active iron protein. The dithionite normally present in a solution of iron protein to prevent oxidative denaturation was consumed enzymatically by addition of MgATP and a trace of molybdenum-iron protein. A known amount of dithionite was introduced and from the drop in absorbance at 430 nm, a value of 0.97 electrons absorbed per active molecule of protein could be calculated. Had a significant amount of inactive protein been present, a lower value would have been obtained. These experiments indicate that normal preparations of iron protein do not contain large amounts of inactive protein, and that the observed low spin integrations may not be rationalized by postulation of such contaminants.

Another hypothesis to explain the low spin-integration, as well as the unusually anisotropic lineshape of the EPR signal, assumes the presence of a second

paramagnet in the protein (24). Such a paramagnet, with lifetime too short to be observed directly, could interact with the observed spin to cause anisotropic broadening and diminution of the signal amplitude. Assumption of this model allowed simulation of the observed signal. Although the postulated second paramagnetic center has not been observed directly (a significant deficiency) this hypothesis is at present the only one to explain the data and to have some supportive evidence.

C. Functional Aspects of MgATP Binding

The binding of MgATP to the iron protein affects many of the latter's physical properties. The accessibility of the iron in the cluster to chelation has been discussed above. In the presence of MgATP, the protein EPR signal reflects a change from rhombic to axial symmetry, with new apparent g values of 2.04 and 1.93 (14,25), while the reduction potential drops to -400 mV (15). Approximately five to six protein thiol groups react with 5,5'-dithiobis(2-nitrobenzoate) in the absence of MgATP, whereas after addition of the nucleotide salt fourteen of the eighteen thiols react (26). This MgATP-induced increase in thiol reactivity was greater than that observed when the protein was denatured in 8M urea! In light of the number of conserved cysteines in the amino acid sequence, and the possibility that the cluster bridges the subunits, it is possible to imagine an MgATP-induced opening of the subunits to expose the buried cluster and cysteine residues to the solvent.

That two molecules of MgATP may bind to each molecule of iron protein has been established by several different types of experiments, some of which also indicate cooperativity in that binding. For example, the rate of dithionite utilization by nitrogenase exhibits a sigmoidal dependence upon MgATP concentration, suggesting cooperativity in binding of the nucleotide salt (K_{d1} = 0.5 μM, K_{d2} = 2.8 μM). A similar dependence was found by measurement of the rate of iron chelation (23), and by observation of the lineshape of the iron protein EPR signal (25) as a function of MgATP concentration. However, when the binding of MgATP to the iron protein was observed directly by gel equilibration (29), no such cooperativity was detected (K_d = 0.4 mM). A rationalization of these disparate conclusions may be found in an examination of the types of experiments on which they were based. Experiments that suggested cooperative binding focussed on characteristics of the iron-sulfur cluster. The MgATP-induced changes in the protein that affect alterations in these properties apparently require binding of both molecules of the nucleotide, thus giving rise to apparent cooperativity. There need be no cooperativity between the binding sites themselves, and indeed

the direct binding study indicates that there is none.

Magnesium ion is required for the ATP-dependent acetylene reduction reaction catalyzed by nitrogenase; however, that reaction is inhibited at high concentrations of Mg^{2+} (30). This is consistent with the requirement of MgATP, and inactivity of Mg_2ATP, in promoting substrate reduction. Either Mg^{2+} or ATP alone has little effect on the chelation of iron from the cluster (23) or the reactivity of protein thiols (26), and is insufficient to elicit the MgATP-induced change in the EPR signal (25). Further, no other nucleotide triphosphate will substitute in any of these reactions.

In the presence of MgADP similar, though not identical, changes occur in the physical properties of the iron protein. For example, the reduction potential of the protein is depressed, although not as much as in the presence of MgATP (15). A change in the EPR signal which somewhat resembles that induced by MgATP occurs when the triphosphate is replaced by the diphosphate (31). Chelation of cluster iron is accelerated by MgADP, though not to the same extent as by comparable levels of MgATP. The diphosphate also serves to inhibit the MgATP-induced acceleration of iron chelation by bathophenanthroline disulfonate (27). Kinetic studies of this inhibition suggest that the iron protein may bind two molecules of either MgATP or MgADP, but is incapable of binding one of each. The observation that the oxidized protein binds MgADP more tightly than it does MgATP, together with the competitive nature of the MgADP inhibition of MgATP binding to the reduced protein, suggests possible in vivo regulation of nitrogenase by the cellular metabolic state (32). This has been examined in vitro in some detail (33).

A stopped-flow kinetic study of dithionite reduction of reversibly oxidized iron protein demonstrated that an initial fast phase ($t_{1/2} < 1$ ms) was associated with reduction of the active protein (34,35). (The rapid reduction was not observed with irreversibly oxidized protein.) Analysis of the data suggested that 0.93 electron equivalents were transferred per mole of protein during this phase. The EPR signal of the native protein was restored completely during this reaction. Although MgATP had no effect on the reduction kinetics, MgADP retarded both the fast reaction and the development of the EPR signal. Thus a basic distinction between the effects of the two nucleotides upon the cluster environment does exist, despite some similarities of their effects upon binding to the protein.

The iron protein from Clostridium pasteurianum forms a tight (K_d = 1 to 10nm) inactive complex with the molybdenum-iron protein from Azotobacter vinelandii (36,37). This complex binds two equivalents of MgATP with a dissociation

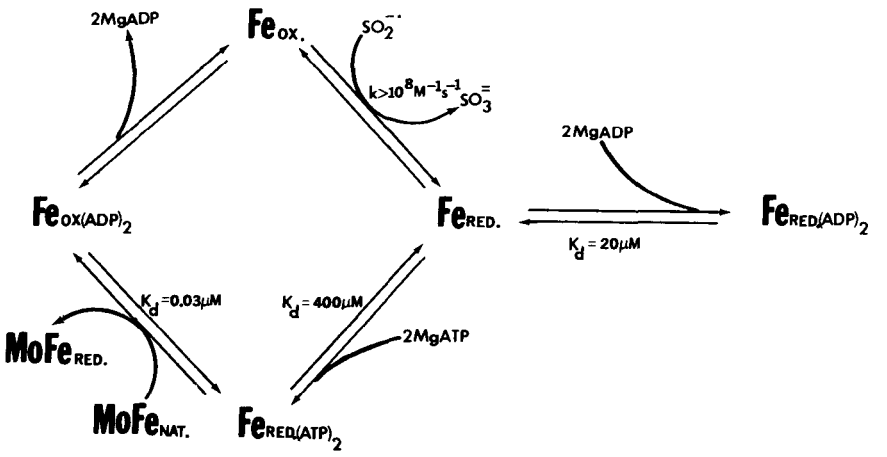

Fig. 1. Reactions of the Iron Protein. The iron protein is reduced by $SO_2^{-\cdot}$ in vitro and oxidized by the molybdenum-iron protein which subsequently reduces substrates. Many of the redox reactions are facilitated by MgATP and hindered by MgADP, as discussed in the text.

constant one-third that of the isolated iron protein. Additionally, the presence of the molybdenum-iron protein protects the iron protein from MgATP-induced chelation of iron from MgATP-induced chelation of iron from the cluster. This suggests that in the complex the electron-carrying iron-sulfur cluster of the iron protein is held buried at the interface between the two proteins, whereas the nucleotide binding sites remain accessible. Thus one of the effects of MgATP may be to expose the cluster to permit a more intimate approach to the electron-acceptor of the molybdenum-iron protein, and thus facilitate electron transfer. Known states of the iron protein in nitrogenase are indicated in Figure 1, along with apparent values for some of the equilibrium constants of interest.

D. Summary

The nitrogenase iron protein is a dimer of identical subunits containing one redox-active tetranuclear iron-sulfur cluster. Both MgATP and MgADP bind to the protein and effect a variety of physically observable changes in the environment of the cluster. MgATP may serve to make electron transfer to the molybdenum-iron protein more favorable thermodynamically by lowering the reduction potential of the cluster, and it may induce more efficient electron transfer by strengthening the binding in the protein complex. There may be a second paramagnet (other than the iron-sulfur cluster) present in the protein, but the present balance of evidence strongly favors a one-electron transfer role for the

iron protein in the nitrogenase complex, coupled to the hydrolysis of 2 moles of MgATP. Binding of MgADP and concomitant inhibition of the overall reaction may lead to regulation of nitrogenase activity in vivo, in response to changes in the cellular MgATP/MgADP ratio.

III. THE MOLYBDENUM-IRON PROTEIN

A. Protein Structure

The molybdenum-iron protein component of nitrogenase has been purified extensively from Azotobacter vinelandii (38,39), Klebsiella pneumoniae (8), Bacillus polymyxa (40), Rhodospirillum rubrum (40), and Clostridium pasteurianum (41,42). From each of these three sources the protein is an $\alpha_2\beta_2$ tetramer of subunits. The α chain molecular weight is reported to be approximately 60,000 while the β chain is approximately 50,000 from Klebsiella and Clostridium and 59,000 from Azotobacter. The total molecular weight of the molybdenum-iron protein is thus 220,000 from Klebsiella and Clostridium and 240,000 from Azotobacter. The quaternary structure of the tetramer is unknown; however, recent small angle neutron scattering studies (43) have shown Cpl to be an ellipsoidal protein with axial ratio of 2:1 and radius of gyration of 39.8 ± 0.7 Å. The proteins from Azotobacter and Clostridium have been crystallized and determination of the structures has begun (44).

In addition to the peptide component, the molybdenum-iron protein contains two gram-atoms of molybdenum, approximately thirty gram-atoms of iron, and approximately thirty gram-atoms of acid-labile sulfur per mole of protein (45). More precise quantitation of iron and sulfur in this labile system is difficult by chemical analysis. The most accurate assignments of composition probably come from the rationalization of spectroscopic experiments. The localization of these iron, sulfur, and molybdenum atoms in individual clusters and investigation of the structure of those clusters constitutes a fair share of the recent progress in the nitrogenase field.

B. Metal Clusters

The molybdenum-iron protein as isolated from every source examined displays a characteristic EPR spectrum with apparent g values near 4.32, 3.65, and 2.01 at temperatures near 4 K (13,45,46,47,48). As the sample temperature is raised above 15 K, the signals rapidly broaden and disappear. These unusual g values may be described adequately as deriving from the ground state Kramer's doublet of an S = 3/2 system (47). Integration of low-temperature EPR spectra of the Azotobacter vinelandii protein gave a spin concentration of 0.91 spins per