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Formation of Ammonia by Insertion of Molecular Nitrogen into Metal–Hydride Bonds. III. Considerations on the Properties of Enzymatic Nitrogen-Fixing Systems and Proposal of a General Mechanism*

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ABSTRACT: Based upon results previously obtained with a nonenzymatic nitrogen-fixing reaction, a general mechanism is proposed for reactions of this type. Double insertion of a N_2 molecule into two metal–hydride bonds of a dimeric μ-hydrido complex, containing the metal in a reduced state, allows for the simultaneous uptake of six reduction equivalents by the N_2 molecule, thus avoiding energetically unfavorable intermediates. If applied to enzymatic nitrogen-fixing reactions, this mechanism would account for hitherto unexplained properties of these systems. In addition, experiments are suggested which would allow proper evaluation of the aptness of this hypothesis.

It is now well established that the reduction of molecular nitrogen to ammonia by aerobic and anaerobic microorganisms occurs through the combined mediation of two nonheme iron particles, one of which contains, besides iron, molybdenum (Bulen, 1966; Mortenson, 1966). Investigations into the dependence of these enzyme systems on various kinds of reducing and phosphorylating agents (Mortenson, 1964; Hamilton *et al.*, 1964; Bulen *et al.*, 1965; Hardy and D'Eustachio, 1964), and into their interaction with inhibitors and pseudosubstrates (Lockshin and Burris, 1965; Schöllhorn and Burris, 1966; Hardy and Knight, 1966) have provided considerable information concerning the nature of the enzymatic reaction paths. To date, however, no satisfactory mechanism has been proposed to account for the observed properties of this interesting reaction. Indeed it is not easy to visualize how the necessary total of six electrons can be transferred from the reducing systems onto the N_2 molecule without the occurrence of energetically very unfavorable intermediates (diimine, etc.). It is the purpose of the present series of publications to draw the attention of the biochemists working in this field, to a coordination-chemical mechanism which could account for the peculiarities of the enzymatic nitrogen reduction, and to demonstrate with suitable model systems the possibility and the conditions of its operation.

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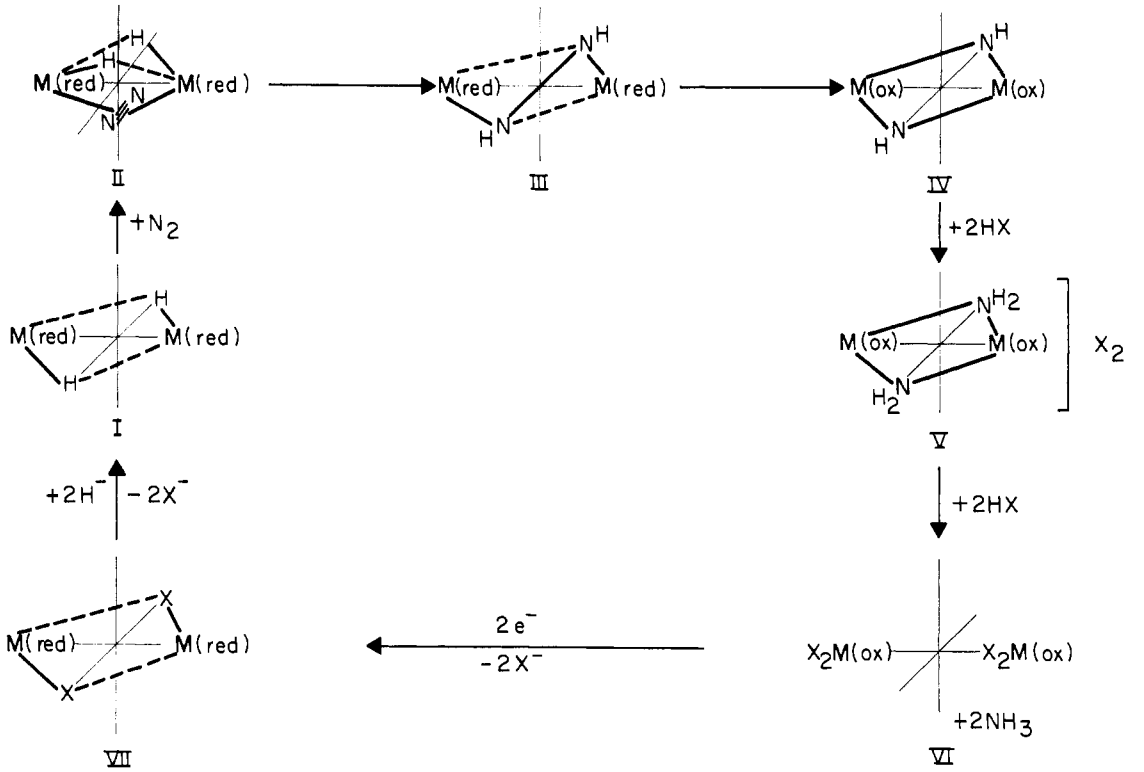
Proposed Mechanism

The N_2 molecule, like other unsaturated systems, should in principle be capable to undergo insertion into metal–hydride bonds (for a review on insertion reactions in metal complexes see Heck, 1964). In fact, an example has been given in preceding publications of this series (Brintzinger, 1966), where such an insertion of N_2 into metal–hydride bonds is very likely to have occurred. It was pointed out that a simultaneous insertion of N_2 into two metal–hydride bonds containing the metal in a reduced state makes indeed possible a very smooth transfer of six reduction equivalents. A catalytic cycle based on this mechanism,



(M(red) = metal in reduced state; M(ox) = metal in oxidized state; HX = acidic reagent)

SCHEME I

as given in the scheme below, might account for many of the features observed with the enzymatic reduction of N_2.

Scheme

The proposed mechanism starts, like that which is supposed to be operative in the nonenzymatic reaction system, from a reduced, dimeric metal hydride in which the hydride ligands act as bridging groups (I) (Scheme I). N_2 is taken up into the complex by formation of two perpendicular bonds to the two metal atoms (II). Only slight nuclear displacements would convert this complex, possibly *via* the reduced, bridged hydrazino derivative III to the bridged di-μ-imino (IV) and -amino (V) complexes, which contain the metal centers in the oxidized state. Two moles of acid are required to release the two ammonia molecules from V (under physiological pH conditions, NH_3 will of course protonate further). The resulting complex VI is then reconverted to the initial hydride I by uptake of two electrons and two hydride ions.

Discussion

1. The Hydride Complex I. Both, molybdenum and iron, are known to form stable metal–hydride bonds in a variety of complexes. The common feature of these complexes is that there have to be present in the complex other π-bonding ligands, typically CO, $C_5H_5^-$, or R_3P. Sulfide ions, which are present in these nonheme iron particles (Bulen, 1966; Mortenson, 1966), and which here, like in other nonheme iron proteins (Hollocher *et al.*, 1966), are most likely to interact with the iron centers, would probably provide a sufficiently π-bonding environment to stabilize metal–hydride bonds.

Quite recently, Bray *et al.* (1966) have observed with another molybdenum-containing nonheme iron enzyme, xanthine oxidase, a proton hyperfine splitting in the α,β-molybdenum electron paramagnetic resonance absorption which might indicate the presence of molybdenum–hydrogen bonds.

Strong evidence for the existence of some sort of hydride in the nitrogenase system is the occurrence of the ATP[1]-dependent hydrogen evolution. In the absence of nitrogen, but in otherwise complete enzyme systems, hydrogen gas is evolved instead of NH_3 (Hardy *et al.*, 1965; Burns, 1965; Burns and Bulen, 1965). It is clear that the scheme given above would explain this observation. If the acidic agent which normally liberates ammonia from complexes IV and V, hits the hydride I instead, which would go unchanged through the first part of the cycle in the absence of nitrogen, H_2 would be released instead of NH_3.

2. The Nitrogen Complex II. There is little doubt that nitrogen forms, in the enzymatic nitrogen fixation

[1] Abbreviation used: ATP, adenosine triphosphate.

reaction, an initial complex with a metal center which resembles in its bonding those of known π-bonding ligands; evidence for this has been derived from the competitive inhibition of the enzyme reaction by CO and NO (Lockshin and Burris, 1965). If the π complex with N_2 as ligand is assumed to be of type II, *i.e.*, to involve two approximately perpendicular bonds of the N_2 π system to two adjacent metal atoms, then it would be easy to understand why the notorious bridge-forming ligand CO competes avidly with N_2 for its binding site (Lockshin and Burris, 1965), in contrast to CN^- which does not normally occur as μ ligand, and which has indeed been reported to interfere with the enzyme reaction only if present in much higher concentrations.[2]

3. The Insertion Reaction. It has been observed (Hardy and Knight, 1966) that the nitrogen-fixing systems of aerobic and anaerobic microorganisms catalyze the reduction of HC≡N according to HC≡N $\rightarrow CH_4 + NH_3$. This reaction is, by analogy to that of the isoelectrionic N_2, to be expected if HC≡N would enter the reaction cycle in the same way as N_2, *i.e.*, by formation of a π complex. Whether HC≡N is capable of undergoing this type of reaction has yet to be shown in suitable model systems; whether the reduction of HC≡N in the enzyme reaction really occurs by an insertion into a metal–hydride bond can be clarified, because of the inertness of CH_4 to proton exchange, by tracer studies. If the above scheme is correct, one hydrogen atom should reach the CH_4 molecule *via* a nonexchangeable protein-bound hydride; the same should hold true for H_2 in the ATP-dependent hydrogen evolution. If, on the other hand, reduction of HC≡N and protons, respectively (and, by analogy, also reduction of N_2) by the enzyme system, would occur *via* an electron transfer from a strong reducing agent, instead of a hydride transfer, all the hydrogen atoms should be derived from the solvent directly.

Acetylene, another compound isoelectrionic to N_2 has been reported to be reduced by nitrogenase systems to ethylene (Schöllhorn and Burris, 1966; Dilworth, 1966; R. Schöllhorn and R. H. Burris, unpublished data). If the complete reaction system was operated in D_2O (so that the metal hydride as well as the acidic reagent would be expected to be present in their deuterated forms) pure *cis*-$C_2H_2D_2$ was formed (Dilworth, 1966). This would be in accord with the formulation of the reduction as an insertion reaction, since the latter are known quite generally to lead to *cis*-reduction products exclusively (Heck, 1964). An explanation for the fact that reduction of acetylene by nitrogenase does not lead further than ethylene could be derived from relative rates of the consecutive insertion and acidolysis reaction steps. Only one of the two hydride moieties can be consumed by this reduction of acetylene; experimentally amenable consequences will be discussed below.

N_2O has been reported to be catalytically reduced by the enzyme systems to N_2 and H_2O (Hardy and Knight, 1966). This reaction deviates, not unexpectedly, from the mechanism described above. The reduced bond is only a double bond and therefore only one hydride is taken up, after which the reaction is interrupted by elimination of N_2. According to the proposed scheme, if only one of the two hydrides is consumed, the acidic reagent involved in steps IV → V → VI should still encounter one hydride per cycle and release it as H_2. In fact it was reported that, unlike in the presence of nitrogen, the ATP-dependent hydrogen evolution is not completely suppressed in the presence of N_2O; instead, H_2 is still released here at about one-half its maximal rate and in an amount comparable to that of N_2 formed from N_2O (Hardy and Knight, 1966). It would be valuable to know whether a similar residual hydrogen evolution occurs also concomitant to the reduction of acetylene to ethylene which, too, as mentioned above, can consume only one of the two metal–hydride bonds.

4. Complexes III–V. Di-μ-imino and -amino complexes of type IV and V have been identified recently as the products of a nonenzymatic nitrogen-fixing reaction (Brintzinger, 1966), but little is known yet about the chemistry of complexes of this type. It is not possible, therefore, to predict, whether all the intermediates III–V will really occur in a catalytic cycle of the proposed kind, or whether one or the other will in fact be by-passed. Also, it is not clear whether the conversion IV → V needs an acidic reagent of appreciable strength already, or whether IV is basic enough to pick up protons from its surroundings. Substantial contributions to these questions can be expected from a detailed investigation of the reaction products of suitable nonenzymatic nitrogen fixation reactions.

5. The Acidolysis Reaction. The behavior of the di-μ-amino complex in the nonenzymatic model reaction suggests, that the release of ammonia will not occur without intervention of a fairly strong acid (Brintzinger, 1966). Transfer of protons onto a substrate is a very common enzymatic reaction step and does not seem to warrant specific remarks. However, consideration should be given to the following possibility. On hydrolysis of ATP protons are released. The parallel between NH_3 formation, respiratory H_2 evolution, and ATP cleavage makes it conceivable that the energetically expensive way of a concerted ATP cleavage is chosen here as a source of protons in order to avoid interference with the formation of the metal–hydride bonds. An investigation of ATP-deficient, purified enzyme systems will certainly clarify this point. If ATP cleavage really were the source of the acidic agent, then the enyzme should accumulate in the absence of ATP in the form of complex IV or V, or, in the absence of nitrogen, in form of the hydride I.

6. Reconversion to the Hydride. Since nothing specific

[2] Two stable complexes with molecular nitrogen as ligand have recently been reported (Allen and Senoff, 1965; Collman and Kang, 1966). It is interesting to note that the Ru(II)–N_2 complex can be reduced by borohydride to form ammonia (Allen and Senoff, 1966).

is known yet about the nature of the hydride form of the enzymes, the question of which role the two enzyme particles play in the conversion of low-potential electrons to hydride ions and their transfer to the proper site of the catalytic reaction, remains completely open for future enzyme–chemical investigations. In subsequent publications the chemical conditions for the operation of the proposed reaction path will be further investigated.

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