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Nitrogenase and nitrogenase reductase associate and dissociate with each catalytic cycle

(nitrogen fixation/hydrogen evolution lag phase/ATP hydrolysis)

ROBERT V. HAGEMAN AND R. H. BURRIS

Department of Biochemistry and Center for Studies of N_2 Fixation, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706

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ABSTRACT **Nitrogenase and nitrogenase reductase dissociate after each electron is transferred between them, as shown by the occurrence of a lag phase approximately as long as the average turnover time of nitrogenase before hydrogen evolution occurs. Because nitrogenase was present in the reaction mixture in large excess over nitrogenase reductase, the electrons donated by nitrogenase reductase must have been distributed randomly over all of the nitrogenase present. This is accomplished by nitrogenase reductase molecules associating randomly with nitrogenase molecules for each cycle of electrons transferred. The fact that ATP is hydrolyzed without a lag indicates both that electron transfer occurs during the lag and that ATP hydrolysis is coupled to electron transfer from nitrogenase reductase to nitrogenase and not to substrate reduction. The observations support the suggestion that it now is desirable to alter nomenclature to designate the MoFe protein as nitrogenase and the Fe protein as nitrogenase reductase.**

The enzyme system responsible for the reduction of N_2 to ammonia has been purified from several organisms and in each case has been found to consist of two proteins (1). The larger protein (molecular weight 210,000–242,000) contains Mo, Fe, and acid-labile sulfur, whereas the smaller protein (molecular weight 55,000–66,000) contains Fe and acid-labile sulfur. Because neither protein alone exhibits any catalytic activity, they frequently are described as forming a catalytically competent complex (2–4). An alternative explanation of the behavior of the two proteins is that the smaller protein serves as a specific reductase for the larger protein, and that the larger protein then reduces N_2 or other substrates. If this be true, the larger protein should properly be called nitrogenase, whereas the smaller protein should be called nitrogenase reductase.* We will present evidence that this nomenclature correctly represents the activity of the two proteins and this nomenclature will be used throughout the paper.

A molecule of nitrogenase reductase binds two ATP molecules (5), and evidence from electron paramagnetic resonance spectroscopy shows that electrons are transferred from nitrogenase reductase to nitrogenase (6). Kinetic studies have indicated that nitrogenase carries the N_2-binding site, whereas nitrogenase reductase acts as a catalytic effector in support of N_2 reduction by nitrogenase (7); Emerich and Burris (8), in their analysis of tight-binding complexes, have treated nitrogenase reductase as a substrate for nitrogenase. However, the question of whether there is a complex of the two proteins that is catalytically active *per se* has remained unanswered. For the complex to serve as a catalytically competent unit, it must remain associated for a time long enough for substrates to be reduced. On the other hand, if the complex exists only long enough for a single electron to be transferred, then it must be inferred that nitrogenase rather than the complex is solely responsible for reduction of substrates after nitrogenase has been reduced by nitrogenase reductase (all reductions by nitrogenase require two electrons or multiples of two electrons).

MATERIALS AND METHODS

ATP, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), Tris, and creatine kinase (EC 2.7.3.2) were obtained from Sigma Chemical Co.; creatine phosphate was from Pierce Chemical Co.; all other chemicals were the highest commercial grade. Nitrogenase and nitrogenase reductase were purified by a modification of the method of Shah and Brill (9). Azotobacter flavodoxin was purified by a modification of the method of Benemann *et al.* (10). Protein concentration was measured by the method of Goa (11), with crystallized bovine serum albumin as the standard. Nitrogenase and nitrogenase reductase concentrations were estimated by accepting the molecular weights assigned by Swisher *et al.* (12). Flavodoxin concentration was estimated by applying the extinction coefficient of 10,600 M^{-1} cm^{-1} at 450 nm for the oxidized form (13). Argon was purified by passage over hot (150°) BASF catalyst R3-11 from Chemical Dynamics Corp. ATP hydrolysis was measured by determining the creatine released (14); the reaction was stopped with saturated K_2CO_3. Evolution of H_2 was monitored continuously by an amperometric method (15). The H_2 electrode was similar in design to a Clark-type O_2 electrode, and the reaction chamber had a volume of 1.7 ml. The electrode was standardized by the addition of an aliquot of buffer saturated with H_2 at 30°.

The reaction mixture contained in 1.0 ml: 50 μmol of Hepes, 25 μmol of creatine phosphate, 5 μmol of magnesium acetate, and 5 μmol of sodium dithionite (unless otherwise indicated). Nitrogenase, nitrogenase reductase, MgATP, and flavodoxin were added as indicated. Reactions were initiated by the addition of either nitrogenase reductase or ATP; the kinetics observed were the same in either case.

RESULTS

Under conditions of very slow electron transfer to nitrogenase there is a significant lag before H_2 is produced (Fig. 1). This lag is independent of how the reaction is initiated, and it cannot be attributed to the response time of the electrode. Evidence from electron paramagnetic resonance spectroscopy suggests that electron transfer between the two proteins occurs during this lag time (16). It is a reasonable assumption that the steady-state rate of H_2 evolution observed after the lag period

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* Nomenclature: The term nitrogenase used in this publication is equivalent to and replaces the terms MoFe protein, component 1, and molybdoferredoxin; whereas the term nitrogenase reductase is equivalent to and replaces Fe protein, component 2, and azoferredoxin.

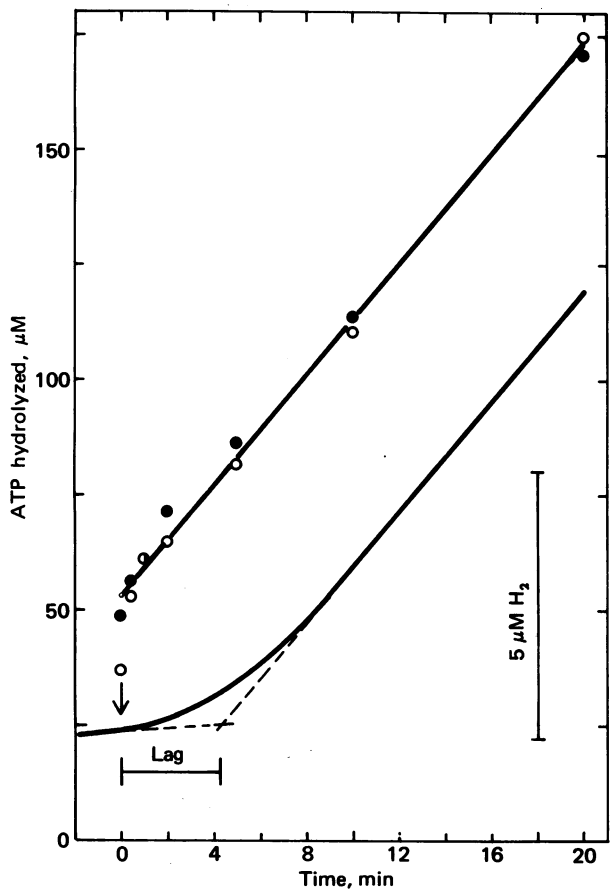

FIG. 1. Progress curves for H_2 evolution and ATP hydrolysis. The reaction mixture contained 6.36 μM nitrogenase, 0.063 μM nitrogenase reductase, and 5 mM dithionite. The reaction was initiated at the arrow by the addition of MgATP to a concentration of 0.50 mM. The lower curve shows H_2 evolution with a 4.3-min lag and a final rate of 0.52 μM H_2 per min. Upper curve (O, ●) shows ATP hydrolysis occurring with no lag and at a rate of 6.0 μM per min. All rates in this work have been expressed as μM min^{-1}; i.e., in terms of change in concentration.

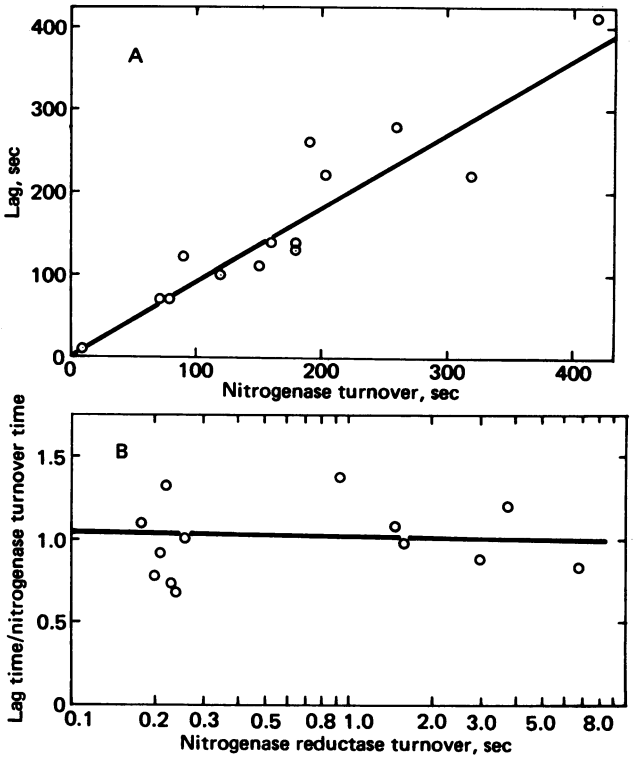

FIG. 2. Lag time as a function of protein turnover times. (*A*) Lag time plotted against nitrogenase turnover time; (*B*) lag time/nitrogenase turnover time plotted against nitrogenase reductase turnover time. The protein turnover times were calculated as described in the text. A plot (not shown) of lag time against nitrogenase reductase turnover time gave a random scattering of points. The data are from Table 1.

represents the rate of electron transfer between the proteins during the lag period. The fact that ATP hydrolysis is linear from the initiation of the reaction (Fig. 1) supports this assumption. This observation provides clear evidence that ATP hydrolysis is required for electron transfer rather than for reduction of substrates.

Table 1 shows how variation of reaction conditions influences electron flux. Fig. 2 shows that the lag period is clearly dependent on the turnover time of nitrogenase, whereas it is independent of the turnover time of nitrogenase reductase. The turnover time of nitrogenase was calculated on the basis of 1 e^- per active site and two active sites per molecule. The turnover time of nitrogenase reductase is based on 1 e^- per molecule (17). The lag period is closely correlated with the nitrogenase turnover time based on these assumptions, and the data indicate that the first electron per nitrogenase molecule is not effective in evolving H_2 but that two electrons must accumulate per molecule to cause H_2 evolution. The calculated number of electrons per active site required for the H_2 evolution is dependent on the purity of the protein and assumptions about the number of active sites. However, the fact that the lag period is proportional to the turnover time is not dependent on any of these assumptions.

DISCUSSION

It is significant that H_2 evolution by nitrogenase occurs after a lag phase proportional to the average turnover time of nitrogenase. This indicates that the electrons donated by nitrogenase reductase are distributed randomly throughout the pool of nitrogenase, even when nitrogenase is present in large excess over nitrogenase reductase. This random distribution is verified by the fact that the rate of H_2 evolution is approximately exponential, as predicted by the model:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} A + H_2$$

This model oversimplifies the H_2-evolving reaction, but it closely approximates the time course observed for H_2 evolution (Fig. 3).

The electrons are distributed randomly over the pool of nitrogenase even at turnover rates of nitrogenase reductase that approach maximal rates (6.7 sec^{-1} for our preparation). Random distribution could occur either by (*a*) direct electron transfer among nitrogenase molecules or (*b*) by distribution of electrons from nitrogenase reductase at random to the pool of nitrogenase molecules. Mechanism *a* is not supported by any experimental evidence, and kinetic and electron paramagnetic resonance experiments indicate that it does not occur (our unpublished data). Mechanism *b* then must be correct, i.e., nitrogenase reductase distributes electrons randomly to nitrogenase. This could not occur if the two proteins formed an active complex that lasted through many electron transfers, so it

Table 1. Lag in H_2 evolution as a function of reaction conditions

	[Dithionite], mM	Flavodoxin, μM	[MgATP], mM	[Nitrogenase], μM	[Nitrogenase reductase], μM	H_2 evolution, μM min^{-1}	Lag time, sec	Nitrogenase turnover, sec	Nitrogenase reductase turnover, sec
1	5	0	0.059	1.87	1.39	11.1	12	10	3.8
2	5	0	0.012	3.74	1.39	1.28	130	180	30
3	5	0	0.029	3.74	0.42	1.82	100	120	6.9
4	5	0	0.059	3.74	0.140	1.38	140	160	3.0
5	5	0	0.294	3.74	0.042	0.85	280	260	1.5
6	5	0	1.18	3.74	0.028	0.54	410	420	1.6
7	5	0	2.9	3.74	0.036	1.76	260	190	0.93
8	5	2.9	2.9	3.74	0.027	3.10	70	70	0.26
9	20	0	2.9	3.74	0.0181	2.51	120	90	0.22
10	5	8.8	2.9	3.74	0.0056	0.71	220	320	0.24
11	5	100	5.0	3.74	0.0070	1.14	220	200	0.18
12	5	59	2.9	3.74	0.0084	1.27	140	180	0.20
13	5	5.9	2.9	3.74	0.0112	1.47	110	150	0.23
14	5	100	2.9	3.74	0.021	2.98	70	75	0.21

follows that the protein complex must dissociate after electron transfer. If the assumptions are valid that were made earlier on the number of active sites, then it is clear that nitrogenase reductase dissociates from nitrogenase after each electron transferred.

We now have evidence that the two proteins, nitrogenase and nitrogenase reductase, dissociate after each electron transfer between them. The lifetime of the complex between the proteins is no longer than the turnover time of nitrogenase reductase. Other work in our laboratory indicates that nitrogenase reductase has no special role in reduction of substrates by nitrogenase other than to reduce nitrogenase. If the lifetime of the complex is only long enough to transfer one electron, the complex *per se* cannot have a direct role in substrate reduction because reduction of all substrates requires multiples of two electrons. Nitrogenase reductase seems to serve solely as a specific reducing agent for nitrogenase, and this reduction is accompanied by the hydrolysis of ATP.

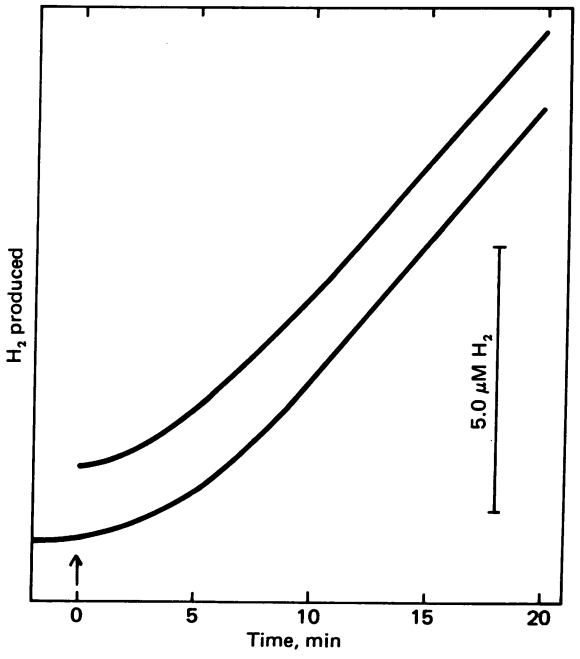

FIG. 3. Comparison of curves for H_2 evolution derived experimentally (lower curve, from Fig. 1) and theoretically (upper curve offset for clarity) as predicted by the model discussed in the text. The equation derived was $H_2 = (v \times t) - A[1 - \exp(-t/\text{lag})]$, in which $v = 0.52$ μM·min^{-1}, A = 2.16 μM, lag = 4.3 min, and t is in min. The constants were chosen to match the experimentally derived constants.

Our experiments have answered two questions relating to the enzymology of the N_2-fixing system. First, nitrogenase is the protein solely responsible for the reduction of substrates, whereas nitrogenase reductase serves only to transfer electrons to nitrogenase. Second, the timing of ATP hydrolysis corresponds to the transfer of electrons between the two proteins and is not directly coupled to substrate reduction.

The evidence presented in this paper indicates that the N_2-fixing system can best be described by calling the proteins nitrogenase (MoFe protein) and nitrogenase reductase (Fe protein). We have used this nomenclature in the present paper and suggest general adoption of the terminology. Systematic names for enzymes must be approved by the IUB Commission on Biochemical Nomenclature, but logical designations would be: for nitrogenase, nitrogenase reductase:dinitrogen oxidoreductase; for nitrogen reductase, donor:nitrogenase oxidoreductase(ATP hydrolyzing).

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