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Purification and Properties of the Constituents of the Nitrogenase Complex from *Clostridium pasteurianum*

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A new procedure for a rapid and extensive purification of the FeMo protein and the Fe protein of the nitrogenase complex from *Clostridium pasteurianum* is described. Specific activities of 345 and 460 nmoles of N_2 reduced per mg of protein per min for the FeMo protein and for the Fe protein, respectively, have been obtained. Preparations of the FeMo protein contained 0.96 atom of molybdenum and 15 atoms of iron per molecule, whereas those of the Fe protein contained 2.86 atoms of iron per molecule. Experiments suggest that a definite association of two Fe proteins and one FeMo protein is functional in the active enzyme complex. No individual role could be ascribed to either of the two proteins, but the fact that hydrogenase inhibits N_2 fixation but not the reductant-dependent adenosine triphosphate hydrolysis supports the idea that there are two distinct sites on nitrogenase, one concerned with N_2 activation and the other with activated electron transport.

Purification of the N_2-fixing systems from *Azotobacter vinelandii* by Bulen and LeComte (2) and from *Clostridium pasteurianum* by Mortenson et al. (17), Moustafa and Mortenson (18), and Kelly et al. (15) has yielded two proteins which are required for N_2 fixation and adenosine triphosphate (ATP)-dependent H_2 evolution. The first protein, containing molybdenum and iron, has been called enzyme I or molybdoferredoxin, and the second, containing iron, has been called enzyme II or azoferredoxin. In this text, these two proteins are referred to as the FeMo protein and the Fe protein, respectively. These designations are explicit and seem advisable until the functions of the individual components are established clearly (1).

This paper describes a rapid procedure for preparing the FeMo protein and the Fe protein from *C. pasteurianum* and discusses the possible involvement of a third component in the clostridial nitrogen-fixing system (14, 16, 22). The relationship between nitrogenase and hydrogenase (26) is examined, and the respective roles of the FeMo and Fe proteins in nitrogen fixation and the question of their association are considered.

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MATERIALS AND METHODS

Cultures. Cultures of *C. pasteurianum* strain W-5 were grown with N_2 in the nitrogen-deficient medium of Westlake and Wilson (25). In the final stage, a 150-liter glass-lined fermentor with medium at 35 C and *p*H 6.4 was used, and phosphates were omitted from the medium. Cells were harvested and dried at 35 C under vacuum as described by Carnahan et al. (3). They were stored at −15 C in evacuated, sealed ampoules.

Extracts. Extracts were prepared by autolysis of dried cells [0.8 to 1.2 g of cells per 10 ml of 20 mM tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.4]. The suspension was shaken under H_2 at 30 C for 1 hr. The crude extract obtained after centrifugation (25,000 × *g* for 20 min) contained 25 to 30 mg of protein per ml. All manipulations at all stages of preparation and purification of the extracts were conducted under strictly anaerobic conditions. All flasks and solutions to be used were evacuated and gassed with H_2 or A three or more times. Transfers between flasks under A, H_2, or N_2 were made with hypodermic syringes through serum stoppers. Whenever a system containing a nitrogenase preparation was open (for introduction of samples on chromatographic columns, during collection of fractions from these columns, during introduction of samples in manometric flasks), a stream of N_2 was kept flushing through the system.

Assays of nitrogenase activity. A typical assay mixture for the determination of nitrogenase activity

contained, for 1 ml of reaction mixture, creatine phosphate, 40 μmoles; creatine phosphokinase, 0.4 mg; ATP, 5 μmoles; $MgCl_2$, 10 μmoles; $Na_2S_2O_4$, 20 μmoles; potassium 2-(*N*-morpholino) ethanesulfonate (MES) buffer (*p*H 6.6), 40 μmoles; and 1 atm of N_2 or A, or 0.4 atm of C_2H_2 plus 0.6 atm of A, or 5 to 10 μmoles of azide and 1 atm of A, according to the assay to be run. Vessels were incubated with shaking (150, 4-cm strokes/min) at 30 C in a water bath for 10 to 15 min. Nitrogen fixation was measured by NH_3 formation as described by Dilworth et al. (6). Acetylene reduction was measured by gas chromatographic determination of the ethylene produced; a Varian Aerograph 600D gas chromatograph with Porapak R column was used as described by Stewart et al. (21). Azide reduction was measured by ammonia formation and manometric measurements. H_2 evolution was measured manometrically. All manometric determinations were performed with Gilson all-glass volumometers (23) which allow convenient evacuation and gassing. The specific activities of the FeMo and Fe proteins are defined as the amount of nitrogen (nanomoles of N_2) fixed per minute per milligram of the specified protein in the presence of an excess of a purified preparation of the complementary protein. The specific activity of nitrogenase is expressed as nanomoles of N_2 fixed per minute per milligram of total protein for a crude extract or for the optimal combination of purified FeMo and Fe proteins in a reconstituted enzyme system.

Analytical methods. ATP hydrolysis was measured by determination of the creatine liberated from creatine phosphate (7). These determinations were performed in the reaction mixtures after the reaction had been stopped by addition of a saturated potassium carbonate solution. Protein was measured by the microbiuret method of Goa (9) with serum albumin as a standard. Molybdenum was determined with the dithiol reagent (4) by the method described by Bulen and LeComte (2). Iron was determined with bathophenanthroline (24). In experiments involving exchange with D_2, determinations of the three molecular species, H_2, HD, and D_2, were made with a Consolidated Nier isotope-ratio mass spectrometer; D_2 served as an internal standard or measurements were made at a standard manifold pressure. Disc electrophoresis of the purified fractions was run in polyacrylamide gels according to Davis (5).

Reagents. All reagents were purchased from commercial dealers, except that creatine phosphate was prepared according to Ennor and Stocken (8). The diethylaminoethyl (DEAE) cellulose used was Whatman DE 52.

RESULTS

Purification of proteins. Crude extracts had a specific activity of about 14 nmoles of N_2 fixed per min per mg of protein. They were first fractionated on an anaerobic DEAE-cellulose column (19). A volume of 60 to 100 ml of extract (1.5 to 2.5 g of protein) was put on top of a DEAE-cellulose column (2.7 cm in diameter by 8 cm in height) which had been equilibrated anaerobically with 0.02 M Tris buffer, *p*H 7.4. The flow rate was 0.8 to 1.0 ml/min. After the extract had entered the column, it was washed with 25 ml of Tris buffer to complete elution of the large quantity (about 50% of total protein) of weakly adsorbed yellowish proteins. Elution then was started with 0.065 M $MgCl_2$ in 0.02 M Tris buffer, *p*H 7.4. This eluted a dark brown band; it was collected as fraction 1 and contained the FeMo protein. The front of this band contained hydrogenase, and the first 6 to 8 ml was collected separately. Elution then was continued with 0.09 M $MgCl_2$ in 0.02 M Tris buffer (*p*H 7.4), and the brown-orange band eluted was called fraction 2. It contained the Fe protein. A dark brown band of ferredoxin was left on the column; it could be eluted with 0.3 M $MgCl_2$. Table 1 gives the results of a representative fractionation experiment.

Fraction 1 contained the FeMo protein and hydrogenase. Hydrogenase was separated by gradient elution on DEAE cellulose. The column (2.9 by 11 cm) was equilibrated with anaerobic 0.02 M Tris buffer, *p*H 7.4. Fraction 1 (usually about 200 mg of protein) was diluted with buffer, so that the final concentration of Mg^{2+} was 0.04 M, and then was applied to the top of the column. Elution was effected with a linear gradient of $MgCl_2$ (0.04 to 0.08 M) in 0.02 M Tris buffer, *p*H 7.4. Two distinct brown bands formed, and fractions were collected in a series of anaerobic bottles. The results of such an experiment are

TABLE 1. *Fractionation of a crude extract on a DEAE-cellulose column*

Determination	Specific activity for N_2 fixation[a]	Protein (mg/ml)	Vol (ml)	Purification (no. of times)
Crude extract	14.5	23.7	79.5[b]	1
Fraction 1		15.5	36	
Residual activity	0			
Supplemented with F_2	76[c]			
Fraction 2		5.6	18.1	
Residual activity	13			
Supplemented with F_1	182[d]			
Reconstituted enzyme	51.3			3.54

[a] Expressed as nanomoles of N_2 fixed/(minutes × milligrams of protein).

[b] A 13.65-g amount of dried cells in 166 ml of 0.02 M Tris, *p*H 7.4, gave 90 ml of extract.

[c] Corrected for the residual activity of the fraction 2 added.

[d] Fraction 1 (F_1) was used to supplement fraction 2 (F_2) in the assay. If, a purified FeMo protein preparation was used instead, the specific activity of the same F_2 was 310.

given in Fig. 1 and Table 2. The first band had the hydrogenase activity, and the second band contained the FeMo protein. The volume of the pooled fractions containing the FeMo protein was reduced by ultrafiltration under N_2 through an Amicon filter.

Immediately after collection from the initial fractionation on DEAE cellulose, fraction 2 was placed on an anaerobic column (2.7 by 33 cm) of Sephadex G-100 equilibrated with 0.02 M Tris buffer (*p*H 8.0), containing 2 mM dithionite and 2 mM dithiothreitol. Two bands were formed when it was eluted with the same buffer. The first band was yellow and contained the Fe protein; the second band contained a red protein. Table 3 gives the results of such a separation. Specific activities of 130 to 140 were obtained for nitrogenase reconstituted from purified FeMo and Fe proteins.

Properties of the constituents of nitrogenase: stability. The FeMo protein concentrated after purification as described was kept in the refrigerator under H_2 for as long as 1 week with a 20% loss of activity. It was kept in liquid nitrogen for longer storage. The Fe protein was much more labile, and a precipitate formed within 1 hr in fraction 2 from the initial DEAE column. All activity was lost in 10 hr unless the preparation was frozen in liquid nitrogen. After purification on Sephadex, the Fe protein was less cold-labile and in general was more stable. No precipitate formed during weeks of storage in the refrigerator, and about 80% of the activity was retained for 2 days in the refrigerator (5 C). If not used immediately, the Fe protein was preserved in liquid nitrogen.

Activity. No preparations of FeMo protein showed any residual activity when assayed alone for reduction of N_2 or other substrates or when assayed for ATP hydrolysis. Fraction 1 evolved H_2 from dithionite independent of ATP because it contained hydrogenase, but purified FeMo protein did not evolve H_2 from dithionite with or without ATP. Fraction 2, however, had residual activity for all nitrogenase reactions. Purification with Sephadex yielded an Fe protein preparation which had no residual activity for N_2 fixation, C_2H_2 reduction, or ATP hydrolysis.

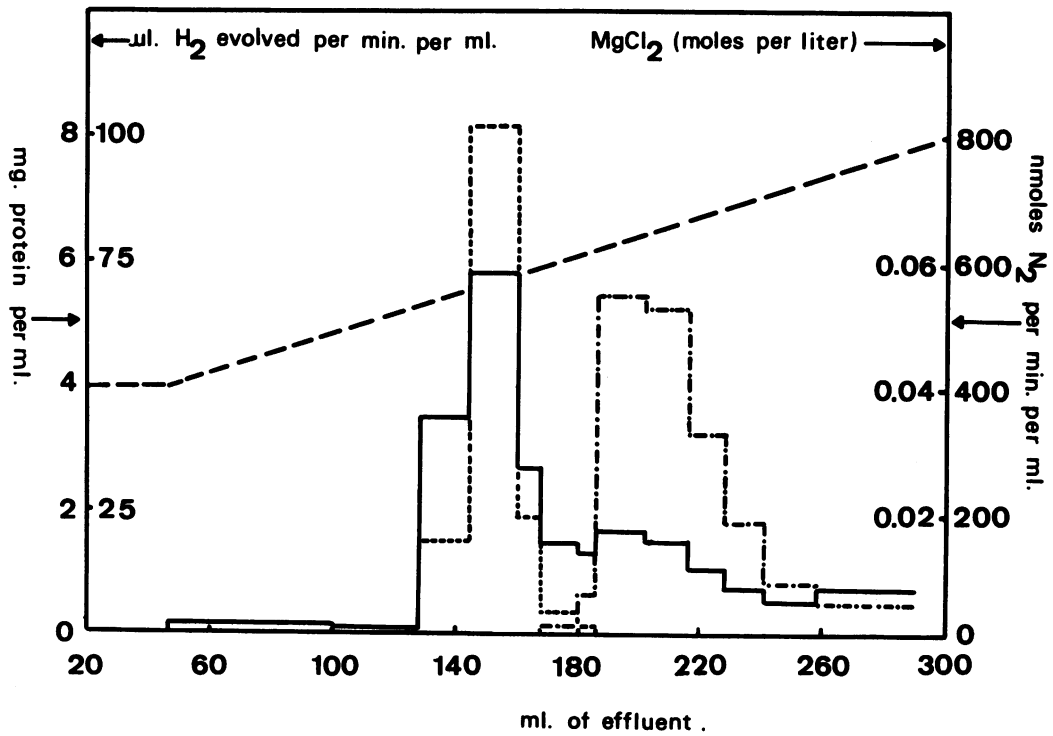

FIG. 1. *Chromatography of fraction 1 by gradient elution from a column of DEAE-cellulose. Symbols: large dashes, $MgCl_2$ concentration; solid line, protein concentration; small dashes, hydrogenase activity; alternating dots and dashes, FeMo protein activity. The hydrogenase activity was measured by H_2 evolution from dithionite at pH 6.6 at 30 C without the addition of an electron carrier. The classical hydrogenase assay of Peck and Gest (20) (in the presence of methyl viologen as an electron carrier) indicated a $Q_{H_2}(N)$ of 6×10^5 for the most active hydrogenase fraction.*

TABLE 2. *Purification of the FeMo protein on a DEAE-cellulose column*

Determination	Vol (ml)	Protein concn (mg/ml)	Specific activity of FeMo protein	Purification (no. of times)
Fraction 1 before purification	20	15.7	76	1
Purified FeMo protein	42.75	1.45	318	4.2

TABLE 3. *Purification of the Fe protein on a column of Sephadex G-100*

Prepn	Vol (ml)	Protein (mg/ml)	Specific activity[a]	
			Residual	Supplemented with FeMo protein[b]
Fraction 2 before purification	10	6.9	20.7	250
Purified Fe protein	11	4.0	0 0[c]	460

[a] Refers to N_2 fixation.
[b] The supplementary FeMo protein preparation had a specific activity of 235 for N_2 fixation.
[c] Refers to C_2H_2 reduction.

Metal analysis of the FeMo and Fe proteins. A purified FeMo protein preparation with a specific activity of 235 had 0.585 μg of Mo per mg of protein or 6.1 nmoles of Mo per mg of protein. Although the reported molecular weight of the FeMo protein has varied between 90,000 and 100,000 (16, 17), the currently accepted value appears to be about 160,000 (1 nmole equals 0.16 mg, or 1 mg of protein is equivalent to 6.25 nmoles). Thus, 6.1 nmoles of Mo per mg of protein corresponds to 0.98 atom of Mo per molecule of the FeMo protein. Analysis for Fe indicated 15 atoms of Fe per molecule. A purified preparation of the Fe protein with a specific activity of 460 contained 2.86 atoms Fe per molecule based on a molecular weight of 40,000 (16). The hydrogenase fraction from the second chromatographic separation on DEAE cellulose had a Q_{H_2} (N) of 6×10^5 and showed no FeMo protein activity, although it carried Fe and a fraction of an atom of Mo per molecule.

Purity. Electrophoretic separations on polyacrylamide gels were run with purified preparations of the FeMo and Fe proteins. The FeMo protein showed two main bands which may be caused by the splitting of the FeMo protein into subunits (17). The Fe protein showed several minor impurities.

Molar ratios of FeMo and Fe proteins in combinations for optimal nitrogenase activity. When nitrogenase was reconstituted by adding increasing amounts of FeMo protein to a given amount of Fe protein, activity per unit of Fe protein increased (Fig. 2), but with excess FeMo protein the specific activity decreased on the basis of total protein of the two fractions. From these data, the respective specific activities of the FeMo and Fe proteins and of the nitrogenase complex can be derived for any combination of the FeMo and Fe proteins. Figure 2 also gives the optimal combination of the FeMo and Fe proteins for nitrogen-fixing activity. A molar ratio approximating 1 FeMo protein to 2 Fe proteins to re-

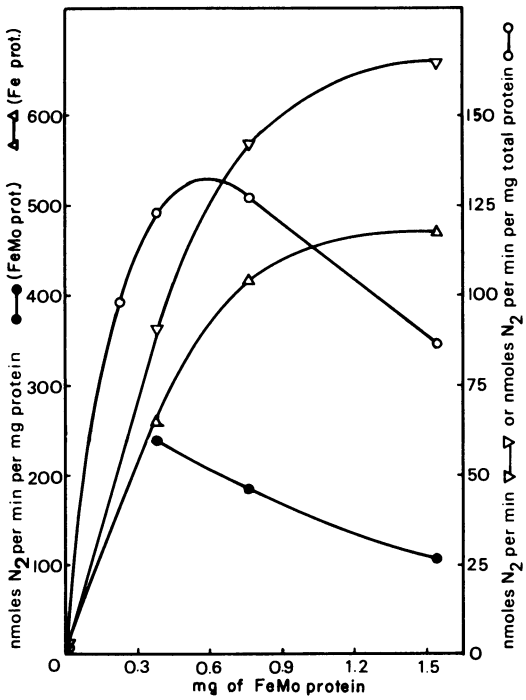

FIG. 2. *Determination of the combinations of the FeMo and Fe proteins giving the optimal specific activities for the FeMo protein, the Fe protein, and the combined total protein. Symbols:* ▽, *nanomoles of* N_2 *fixed per minute;* ●, *specific activity for the FeMo protein;* △, *specific activity for the Fe protein;* ○, *specific activity for nitrogenase. A 0.35-mg amount of Fe protein was added to each vessel, and amounts of FeMo protein were added as indicated on the x axis. The left y axis indicates specific activities (nanomoles of* N_2 *per minute per milligram of protein) based upon the FeMo protein* (●), *or upon the Fe protein only* (△) *with specified amounts of FeMo protein present. The right y axis indicates nanomoles of* N_2 *reduced per minute* (▽) *and the specific activity based on total protein (Fe protein plus FeMo protein,* ○).

constitute the N_2-fixing complex was indicated by applying molecular weights of 160,000 and 40,000, respectively, and by correcting for the estimated purity of the proteins. (Experiment 1, 0.77 mg of 80% pure FeMo protein and 0.35 mg of 95% pure Fe protein were combined; 0.62/0.16 mg per nmole = 3.87 nmoles; 0.33/0.04 = 8.25 nmoles; 8.25/3.87 = 2.13 molar ratio of Fe protein to FeMo protein. Experiment 2, 0.92 mg of 80% pure FeMo protein and 0.40 mg of 95% pure Fe protein were combined; 0.74/0.16 = 4.63 nmoles of FeMo protein; 0.38/0.04 = 9.5 nmoles of Fe protein; 9.5/4.63 = 2.05 molar ratio of Fe protein to FeMo protein.)

Inhibition of N_2 fixation by hydrogenase. The fractions derived from separation of the crude extract on DEAE-cellulose were assayed in the way illustrated in Fig. 2. If fraction 1 instead of purified FeMo protein was used to complement fraction 2, lower values were obtained for fraction 2 activities. In addition, the curve for fraction 2 specific activity instead of reaching a plateau when larger amounts of fraction 1 were added showed a maximum followed by a decrease. Figure 3 illustrates this fact and also shows that ATP hydrolysis, in contrast to N_2 fixation, is not inhibited by excess fraction 1. The same phenomenon was observed when C_2H_2 reduction instead of N_2 fixation was measured. If the complementary experiment was run (measurement of N_2 fixation and ATP hydrolysis when increasing amounts of fraction 2 were added to a constant amount of fraction 1), a normal saturation curve was obtained. Because the inhibition of N_2 fixation was no longer observed when a purified FeMo protein preparation was used to complement fraction 2, it appeared likely that hydrogenase caused the inhibition. This was verified by observing the effect of the addition of increasing amounts of a purified hydrogenase preparation to a given mixture of the FeMo protein and fraction 2 protein. N_2 fixation was strongly inhibited but ATP hydrolysis was not (Fig. 4). A possible explanation is that hydrogenase is able to accept electrons from nitrogenase by an ATP-dependent process. Experiments reported below on H_2 evolution are in agreement with this view.

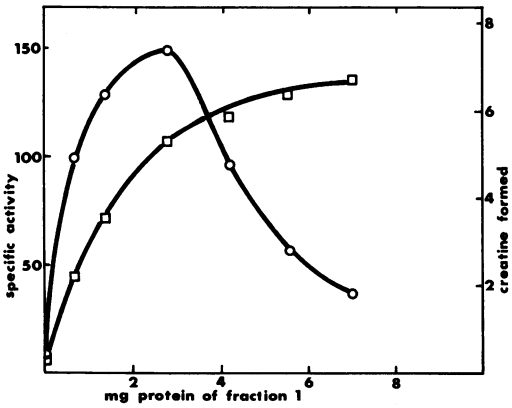

FIG. 3. *Inhibition of N_2 fixation by adding increasing amounts of fraction 1 to a constant amount of fraction 2. The fraction 1 used had a specific activity of 44. Specific activity of fraction 2 (○) is shown on the left y axis; the amount (μg) of creatine formed per minute per milligram of protein of fraction 2 (□) is shown on the right y axis.*

Experiments on H_2 evolution. The hydrogenase fraction obtained by the gradient elution of fraction 1 from a DEAE-cellulose column was free from demonstrable ferredoxin and the FeMo protein, but still evolved H_2 from dithionite at a reduced rate. Addition of the FeMo or Fe protein to a hydrogenase preparation, in the presence or the absence of ATP, did not enhance this H_2 evolution. This shows that neither the FeMo nor Fe protein is able to act alone as an electron carrier (dependent or independent of ATP) from dithionite to hydrogenase. However, when the ATP-dependent H_2-evolving system, consisting of the FeMo and Fe proteins plus an ATP-gen-

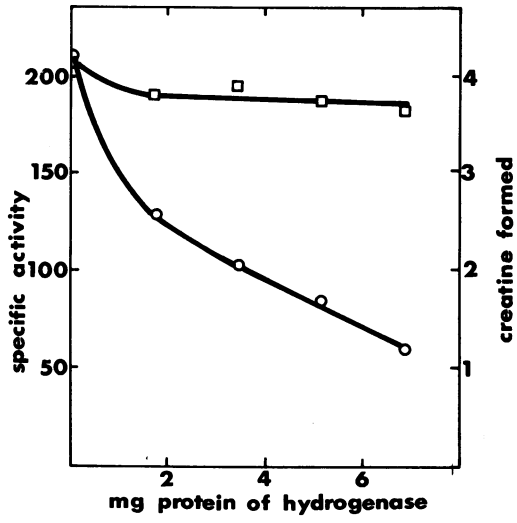

FIG. 4. *Inhibition of N_2 fixation by hydrogenase. The reaction mixture contained, in addition to the standard components in 1 ml: hydrogenase ($Q_{H_2}(N)$ = 5 × 10^5), 0.75 mg of FeMo protein (specific activity, 210), and 0.5 mg of protein of fraction 2 (specific activity, 245). Dithionite was 40 instead of 20 mM. The time of incubation was 8 min. Specific activity, expressed as nanomoles of N_2 fixed per minute per milligram of fraction 2 protein (○), is shown on the left y axis; amount (μmoles) of creatine formed per minute per milligram of fraction 2 protein (□) is shown on the right y axis.*

erating system (Fig. 5), was added to a hydrogenase preparation evolving H_2 from dithionite independent of ATP, the H_2 evolution was only slightly increased, and total H_2 evolution was much less than the sum of the two H_2-evolving systems measured separately. This suggests that, under these conditions, hydrogenase may function as an electron acceptor from nitrogenase.

DISCUSSION

Initial fractionation of nitrogenase into its two proteins markedly shortens the purification procedure. One quickly obtains both proteins from the same crude extract and can apply suitable purification steps to each independently. The method yields a purified Fe protein preparation in 10 to 12 hr after starting with dried cells. The purified FeMo protein requires 16 hr of preparation, but the procedure can be interrupted after the initial fractionation step. In several preparations of the FeMo protein, hydrogenase contamination could be detected. Hence, further purification on a DEAE-cellulose or on a Sephadex G-200 column may be desirable. No evidence was found for the involvement of a third component in the nitrogen-fixing system from *C. pasteurianum*, and the requirement for a third component appears unlikely at the stage of purification attained (14, 16, 22).

Hydrogenase at high concentrations inhibits N_2 fixation but not ATP hydrolysis; this suggests that hydrogenase (CO sensitive) competes with the N_2-fixing system for the ATP-dependent electron flow. As reported by Winter and Burris (26), addition of a complete ATP-dependent H_2-evolving system to a preparation of hydrogenase produces only a small enhancement in H_2 evolution instead of the large increase expected if the systems are running independently. It is clear, however, that both the FeMo and Fe proteins are required for electron donation to produce H_2 by the ATP-dependent route. This can be interpreted as a donation of electrons from the reduced-activated nitrogenase complex

The scheme presented in Fig. 6 indicates the relationship between hydrogenase and nitrogenase. In this scheme, X and Y, respectively, represent the reductant site and the N_2 binding site of nitrogenase proposed by Hardy et al. (11) and discussed in a recent review (10). The evidence for the existence of two distinct sites is based mainly upon the effects of CO on nitrogenase. Because N_2 fixation is inhibited by CO but ATP-dependent H_2 evolution is not, it is logical to assume that CO binds to site Y. Thus, in the presence of CO, site X will evolve H_2 by

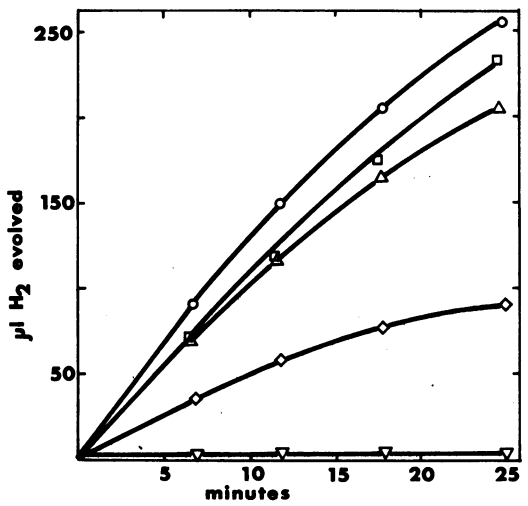

FIG. 5. *Hydrogenase and ATP-dependent H_2 evolution. Each flask contained, in a volume of 1 ml: dithionite, 40 mM; MES (pH 6.6), 40 mM; and, when indicated, the standard ATP-generating system, 1.7 mg of hydrogenase, 0.56 mg of FeMo protein (specific activity, 210), and 0.5 mg of protein of fraction 2 (specific activity, 245). Addition of either FeMo protein or fraction 2 to hydrogenase (not represented here) did not increase the H_2 evolution. Symbols: ○, hydrogenase; □, hydrogenase, FeMo protein, fraction 2, ATP; △, hydrogenase, ATP ◇, FeMo protein, fraction 2, ATP; ▽, FeMo protein, ATP.*

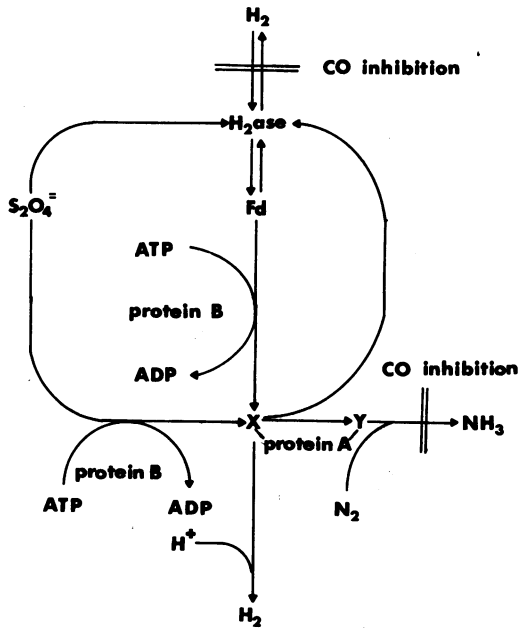

FIG. 6. *Electron flow between hydrogenase and N_2 and scheme for N_2 fixation in Clostridium pasteurianum.*

an ATP-dependent process instead of transferring electrons to site Y. The residual ATP-dependent H_2 evolution in the presence of N_2 may also be interpreted as evidence for the existence of two sites, but alternative explanations are conceivable. For example, this H_2 evolution could result from a reaction at the site of N_2 binding analogous to the addition of N_2 on metal hydride complexes of the type $CoH_3(PPh_3)_3 + N_2 \rightleftharpoons CoH(N_2)(PPh_3)_3 + H_2$. The inhibition by hydrogenase of N_2 fixation but not of ATP hydrolysis provides new evidence for the existence of two different enzymatic sites; the inhibition can be attributed to the ability of site X to transfer its electrons to hydrogenase instead of donating them to site Y.

Finally, the fact that any manifestation of nitrogenase activity requires both the FeMo and Fe proteins can be mechanistically expressed in several fashions. In the scheme shown (Fig. 6), sites X and Y have been located on the same protein A which may be either the FeMo or Fe protein. The other protein, B in the scheme, is depicted as mediating the activation of reduced X through ATP hydrolysis.

The role of ATP in the nitrogenase reactions is not understood. The ATP/2*e* ratios, as measured for N_2 fixation and H_2 evolution, were found between 4.5 and 5; this is in reasonable agreement with recent reports (2, 26; K. L. Hadfield and W. A. Bulen, 1968, Fed. Proc. **27**: 593). N_2 inhibited the ATP-dependent H_2 evolution by 65%, but the overall activity of the nitrogenase complex was unchanged. The determination of the ATP/2*e* ratio for azide reduction was more difficult because of the complexity of the reaction. With an azide concentration of 5 to 10 mM and a gas-to-liquid ratio of 15 to 1, much of the azide reduced was not released as N_2 but was further reduced, suggesting that it remained bound for direct reduction. Experiments with acetylene suggested that the ATP/2*e* ratios may be below 4 for C_2H_4 production. However, there is insufficient evidence yet to conclude whether the ATP/2*e* ratios differ for various substrates, and definitive information on this point may aid in interpreting the mechanism of action of ATP.

The individual role of each protein and the location of the N_2 binding site are of particular interest. H_2 is a competitive inhibitor of N_2 fixation, and there are indications that an enzyme-bound nitrogen intermediate is responsible for the formation of HD from D_2 in reactions mixtures which fix N_2 (12, 13); hence it was of interest to determine whether HD production required the FeMo or Fe protein or both. However, little HD production from D_2 was found under N_2 or A in the presence of dithionite, the ATP-generating system, and the FeMo or Fe protein or their combinations.

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