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ATP HYDROLYSIS AND ELECTRON TRANSFER IN THE NITROGENASE REACTION WITH DIFFERENT COMBINATIONS OF THE IRON PROTEIN AND THE MOLYBDENUM-IRON PROTEIN

TORBJØRN LJONES AND R. H. BURRIS

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

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SUMMARY

Purified iron protein (Fe protein) and molybdenum–iron protein (Mo–Fe protein) of nitrogenase from *Clostridium pasteurianum* were used for studies of ATP hydrolysis and electron transfer in the nitrogenase reaction. Total electron transfer was determined by measuring dithionite oxidation spectrophotometrically. Comparison with manometric determination of H_2 evolution showed that dithionite serves as a two electron donor. The studies of different combinations of the Fe protein and the Mo–Fe protein showed that excess Mo–Fe protein inhibited electron transfer but not ATP hydrolysis, whereas excess Fe protein did not inhibit either reaction. The ATP:2 electron ratios ranged from around 4 with excess Fe protein to more than 20 with excess Mo–Fe protein. With relatively low levels of Mo–Fe protein, reductant-independent ATP hydrolysis was around 6 % of the reductant-dependent value, and with a large excess of Mo–Fe protein it increased to about 10 %. We conclude that because the ATP:2 electron ratio varies with experimental conditions, no single value can be ascribed to this ratio.

INTRODUCTION

Nitrogenase is responsible for reduction of N_2 to ammonia and also can catalyze a number of other reductions, among them the reduction of protons to H_2 and acetylene to ethylene. All these reactions require ATP[1] in addition to a reductant. Nitrogenase catalyzes hydrolysis of ATP to ADP and orthophosphate[2,3]. The quantitative relationship between ATP hydrolysis and electron transfer has been studied by a number of investigators, but no general agreement has been reached. Most workers support a requirement of 4–5 ATP molecules per pair of electrons transferred (ATP:$2e^-$ ratio)[3–6], but Hardy and Knight[2] and Jeng *et al.*[7] have reported ATP:$2e^-$ ratios of 2. Hadfield and Bulen[8] found that the ATP:$2e^-$ ratio depends upon temperature, increasing from 4.3 at 20 °C to 5.8 at 40 °C. They suggested that ATP is involved in at least two reactions with different activation energies, one leading to electron transfer accompanied by ATP hydrolysis and another effecting only ATP hydrolysis.

Abbreviations: BES, *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid; Mo–Fe protein, the molybdenum–iron protein of nitrogenase; Fe protein, the iron protein of nitrogenase.

Most studies of the ATP:$2e^-$ ratio have been done with partially purified nitrogenase preparations in which the Fe protein and the Mo–Fe protein have not been separated. Jeng *et al.*[7] isolated the individual proteins but used only one recombination ratio in their studies of stoichiometry. It appeared possible that differences in the relative amounts of Fe protein and Mo–Fe protein could account for discrepancies in the reported stoichiometry. This paper describes experiments which show that variation of the Mo–Fe protein:Fe protein ratio affects the rates of electron transfer and ATP hydrolysis differently.

MATERIALS AND METHODS

Cultures and enzymes

Cultures of *Clostridium pasteurianum* strain W-5 were grown, and cell-free extracts were prepared as described earlier[9]. Further treatment of the extracts consisted of precipitation with polyethylene glycol, chromatography on DEAE-cellulose, chromatography of the Mo–Fe protein on Sephadex G-200, and chromatography of the Fe protein on Sephadex G-100. The extracts were kept under an atmosphere of N_2 or H_2 throughout the isolation procedure. Details of the purification procedure will be published elsewhere. The proteins appeared essentially pure when tested by anaerobic polyacrylamide gel electrophoresis (M.-Y. Tso, T. Ljones and R. H. Burris, unpublished).

Analytical methods

Dithionite oxidation was measured spectrophotometrically[10]. Cuvets with a 10-mm light path were closed with rubber serum stoppers; they permitted measurements in a total volume of 1 ml. The solutions in the cuvets were made anaerobic by sparging with purified N_2 through hypodermic needles for 30 min. Absorbance was recorded continuously with a Gilford recording spectrophotometer. Nitrogen fixation assays were performed as described previously[9], except that the indophenol reaction[11] was used for ammonia determination after microdiffusion. H_2 evolution was measured manometrically with Gilson all-glass volumometers[12]. Phosphate released during ATP hydrolysis was determined with the method of Rathbun and Betlach[13]. Protein was determined with the microbiuret method of Goa[14] with crystalline bovine serum albumin as a standard.

Chemicals

High-purity N_2, H_2 and Ar were purified by passing them over BTS catalyst (BASF Colors and Chemicals Inc., 866 Third Ave., New York City) heated to about 120 °C. The sodium dithionite used was from Hardman and Holden Ltd., Manchester, England. *N,N*-Bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) buffer was obtained from Schwartz/Mann. Creatine phosphate was obtained from Pierce Chemical Company. Creatine kinase and ATP (Sigma grade disodium salt) were obtained from Sigma Chemical Company.

RESULTS

Stoichiometry between dithionite oxidation and H_2 evolution

We have reported previously that spectrophotometric measurement of dithionite

oxidation can be used as an assay for total electron transfer in the nitrogenase reaction[10]. We expected that dithionite would act as a two-electron donor and produce sulfite[15] during the nitrogenase assays. There is no report that sulfite can act as a reductant in nitrogenase-catalyzed reactions, however, because the method was proposed for studies of stoichiometry, dithionite oxidation was compared with manometrically measured evolution of H_2 from a specific amount of dithionite. The concentration of the dithionite stock solution was determined spectrophotometrically after appropriate dilution with buffer (extinction coefficient of $Na_2S_2O_4$ is 8000 $M^{-1} \cdot cm^{-1}$ at 315 nm [15]).

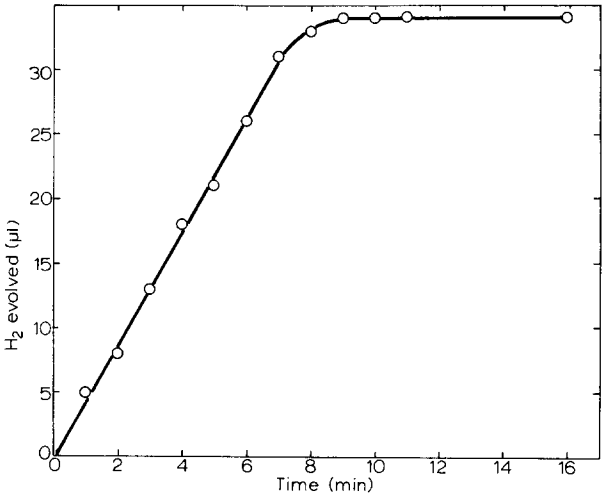

Fig. 1. Time course of H_2 evolution. Evolution of H_2 was determined manometrically under Ar; see text. The incubation mixture contained: 5 mM ATP, 40 mM creatine phosphate, 0.4 mg/ml creatine kinase, 10 mM $MgCl_2$, 25 mM BES buffer, pH 7.0. All ingredients except creatine kinase were adjusted to pH 7.0 with 1 M KOH; the total volume was 3.0 ml. 1.86 μmoles $Na_2S_2O_4$ were added before the enzyme, which consisted of 0.32 mg Fe protein and 0.28 mg Mo–Fe protein. The temperature was 30 °C.

The time course of the evolution is shown in Fig. 1. The curve is linear for most of the period of H_2 evolution, and breaks rather sharply before H_2 evolution ceases. This pattern is similar to that of dithionite oxidation measured spectrophotometrically[10]; in each case the rates are constant over large changes in dithionite concentration. Spectrophotometric measurement of dithionite oxidation with the same amounts of enzyme and dithionite designated in Fig. 1 showed that H_2 evolution and dithionite oxidation ceased after the same time.

Table I shows the results of two sets of measurements of H_2 evolution. Although there are relatively large variations in the molar ratios of H_2 evolved and $Na_2S_2O_4$ oxidized, the results nevertheless support a whole number ratio of 1. $Na_2S_2O_4$ is autooxidizable, so small amounts of contaminating air will cause errors.

Fig. 1 shows no slow continuation of H_2 evolution after the dithionite has been exhausted. Another experiment showed no additional evolution of H_2 for 15 more min. Hence, the oxidation products of dithionite, probably sulfite, do not support a slow continuation of the reduction, unlike some other systems[15].

TABLE I

EVOLUTION OF H_2 FROM DITHIONITE

Conditions for manometric determination of H_2 evolution are described in the legend to Fig. 1. Amounts of enzyme proteins were: Expt I, 0.64 mg Fe protein and 0.56 mg Mo–Fe protein; in Expt II, 0.32 mg Fe protein and 0.28 mg Mo–Fe protein. Values for amounts of dithionite were corrected for decomposition at pH 7.0 (ref. 10).

Expt	*μmoles* $Na_2S_2O_4$	*μmoles* H_2	$\frac{\mu moles\ H_2}{\mu moles\ Na_2S_2O_4}$
I	3.3	3.2	0.97
	2.5	2.4	0.95
	3.1	3.5	1.13
II	1.80	1.31	0.73
	1.78	1.54	0.88
	1.75	1.73	0.99

Activities of the individual components of nitrogenase

The purified preparations of Fe protein and Mo–Fe protein were tested individually for N_2 fixation, dithionite oxidation and ATP hydrolysis. No activity could be detected in any of the tests. This shows that the preparations contain no contaminating ATP hydrolysing activity (ATPase). Nitrogenase has some ATPase activity in the absence of reductant (discussed below), and the presence or absence of a contaminating ATPase therefore can be examined only with purified Fe protein or Mo–Fe protein that individually is free of residual nitrogenase activity. The remote possibility exists that the Mo–Fe protein and the Fe protein preparations each contain a contaminating ATPase component (not nitrogenase) which together form an active ATPase.

Change in the rates of dithionite oxidation and ATP hydrolysis with time

Nitrogenase assays in the absence of an ATP-generating system such as creatine phosphate and creatine kinase show deviation from linear time courses, probably because of inhibition by ADP[3]. For studies of the stoichiometry between ATP hydrolysis and electron transfer, we chose to omit creatine phosphate because of the complications this labile compound introduces in the phosphate analysis. Therefore, it was important to know whether or not the proportional decrease in reaction rates with time is the same for ATP hydrolysis and electron transfer. Fig. 2 shows a proportional decrease. The random variation in the ATP:$2e^-$ ratio appears to arise from experimental error. Because it is difficult to obtain true initial velocities with the fixed time assay for ATP hydrolysis, we have in further experiments corrected for ADP inhibition by comparing ATP hydrolysis with initial and overall rates for dithionite oxidation.

ATP:$2e^-$ ratios at different levels of Fe protein and Mo–Fe protein

Experiments were performed in which the amount of one of the two protein components was constant and the other was varied, and the rate of electron transfer was determined by spectrophotometric measurement of dithionite oxidation. The reaction was terminated with trichloroacetic acid when about 50 nmoles of dithionite had been oxidized, and the mixture was analyzed for inorganic phosphate. The ATP:$2e^-$ ratios were calculated from the phosphate analysis and the spectrophoto-

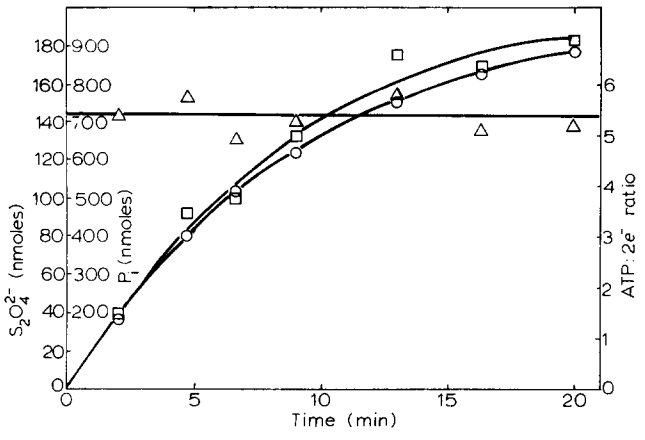

Fig. 2. Time course of ATP hydrolysis and of dithionite oxidation. The reaction mixture contained: 5 mM ATP, 5.8 mM $MgCl_2$, 0.3 mM $Na_2S_2O_4$ and 20 mM BES buffer, pH 7.0. (The mixture of ATP and $MgCl_2$ was adjusted to pH 7.0 with 1 M KOH.) The total volume was 1 ml, and the temperature was 25 °C. The reaction was started simultaneously in two cuvets by injection of the enzyme solution, which contained 0.20 mg Fe protein and 0.14 mg Mo–Fe protein. Dithionite oxidation was followed spectrophotometrically at 315 nm in one of the cuvets. Samples were withdrawn from the other cuvet and mixed with trichloroacetic acid to a final concentration of 5%. Phosphate was determined as described in Materials and Methods. ○—○, dithionite oxidized; □—□, phosphate released; △—△, ATP:$2e^-$ ratio.

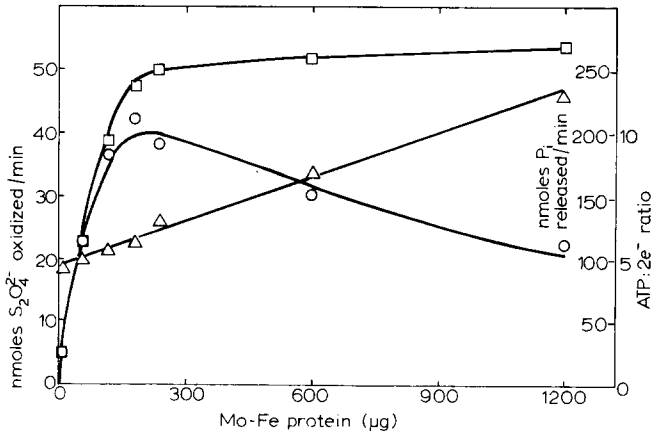

Fig. 3. ATP hydrolysis and dithionite oxidation at different levels of the Mo–Fe protein. The conditions were as described in the legend to Fig. 2. The dithionite concentration was 0.1 mM. After oxidation of about 50 nmoles of dithionite, the reaction was stopped by addition of 65 μl of trichloroacetic acid, 0.8 g/ml, and analyzed for phosphate. All reaction mixtures contained 0.18 mg Fe protein. ○—○, rate of dithionite oxidation; □—□, rate of ATP hydrolysis; △—△, ATP:$2e^-$ ratio.

metric determination of the total amount of dithionite oxidized in the same time span. Figs 3 and 4 record the results of two such sets of experiments. Fig. 3 shows that excess Mo–Fe protein inhibits dithionite oxidation. Inhibition by excess Mo–Fe protein was also found by Shah *et al.*[16] and Mortenson[17]. Excess Mo–Fe protein did not inhibit ATP hydrolysis.

Fig. 4 shows no inhibition of dithionite oxidation or ATP hydrolysis by excess

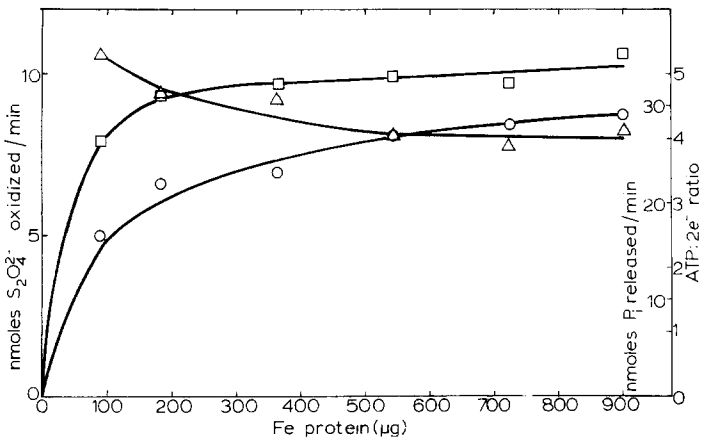

Fig. 4. ATP hydrolysis and dithionite oxidation at different levels of the Fe protein. Same conditions and symbols as in the legend for Fig. 3. Each reaction mixture contained 0.012 mg Mo–Fe protein.

Fe protein. The ATP:$2e^-$ ratio increases with increasing amounts of Mo–Fe protein, but with increasing amounts of Fe protein it reaches a constant value near 4. The highest value with excess Mo–Fe protein (Fig. 3) is 11.5, but in other experiments values of more than 20 have been obtained.

The ATP:$2e^-$ ratios were not corrected for reductant independent ATP hydrolysis as it was catalyzed by nitrogenase and not by some contaminating ATPase.

Reductant-independent ATP hydrolysis

Bui and Mortenson[18] and Jeng *et al.*[7] found a high level of ATP hydrolysis in the absence of reductant, and the reductant-independent hydrolysis was more active relative to the reductant-dependent hydrolysis at low pH. Representative calculations from their graphs for reductant-independent ATP hydrolysis as percentages of total ATP hydrolysis are: 82 % at pH 6.0 and 54 % at pH 7.0[18] and 53 % at pH 6.0 and 33 % at pH 7.0[7]. In each case purified Fe protein and Mo–Fe protein from *C. pasteurianum* was used. Hadfield and Bulen[8] used a purified, particulate nitrogenase preparation from *Azotobacter vinelandii* and found only 5 % reductant-independent ATP hydrolysis at pH 7.25. In these and other reports, the ATP:$2e^-$ ratios were corrected for reductant-independent ATP hydrolysis.

We compared rates of ATP hydrolysis in the presence and absence of dithionite under the conditions described in the legends for Figs 3 and 4; 180 μg of Fe protein and 120 μg of Mo–Fe protein were used. The rate in the presence of dithionite was 198 nmoles P_i released/min and in the absence of dithionite it was 12 nmoles P_i released/min, *i.e.* 6 % of the reductant-dependent hydrolysis.

In another experiment, with an initial concentration of 0.01 mM dithionite, the reaction was followed spectrophotometrically until all the dithionite was oxidized whereupon a sample was withdrawn for phosphate analysis. The rest of the mixture was incubated an additional 10 min before another sample was taken for phosphate analysis. After depletion of dithionite, the rate of ATP hydrolysis as calculated from the difference in phosphate content was 20 nmoles/min, *i.e.* 10 % of the reductant-

TABLE II

REDUCTANT-DEPENDENT AND REDUCTANT-INDEPENDENT ATP HYDROLYSIS AT TWO DIFFERENT RATIOS OF NITROGENASE PROTEINS

Conditions were as described for Figs 3 and 4. Each reaction mixture contained 0.14 mg Fe protein. The incubation time was 10 min when dithionite was omitted.

Mo–Fe protein (mg)	*$Na_2S_2O_4$ (mM)*	*Rate of $S_2O_4^{2-}$ oxidation (nmoles/min)*	*Rate of ATP hydrolysis (nmoles/min)*	*ATP:$2e^-$ ratio*
0.14	0.1	61.2	420	7
0.14	0		25	
1.12	0.1	23.8	460	19
1.12	0		44	

dependent value. This is somewhat higher than found without any exposure to dithionite, but is much lower than reported by Mortenson and coworkers[7,18].

Reductant-independent and reductant-dependent ATP hydrolysis were compared at two different combination ratios between Fe protein and Mo–Fe protein (Table II). A large excess of Mo–Fe protein gave a rather small increase in the reductant-independent ATP hydrolysis, an increase entirely inadequate to account for the large differences in the ATP:$2e^-$ ratio observed with changing protein ratios.

DISCUSSION

Our observation that the ATP:$2e^-$ ratio is a function of the relative amounts of the Fe and the Mo–Fe protein provides further evidence for Hadfield and Bulen's[8] suggestion that ATP is hydrolyzed by nitrogenase in at least two different reactions, and consequently no whole number reaction stoichiometry can be ascribed to the nitrogenase-catalyzed ATP hydrolysis and electron transfer. Discrepancies among the published values for the ATP:$2e^-$ ratio may arise from use of different levels of the two proteins. Different levels could be present even in unfractionated enzyme preparations, because one of the two components may have been inactivated more extensively than the other or have been selected against during isolation. Inactivation by oxygen could alter the ratio between active components during isolation, because the Fe protein is more easily inactivated by oxygen than the Mo–Fe protein.

Jeng *et al.*[7] found rather high activity for reductant-independent ATPase and suggested that differences in the published values for the ATP:$2e^-$ ratio could be attributed to ignorance of the existence of reductant-independent ATP hydrolysis. They contended that the ATP:$2e^-$ ratio obtained when the reductant-independent ATP hydrolysis is subtracted from hydrolysis in the presence of reductant furnishes the correct basis for calculating stoichiometry for that part of the ATP consumption which is coupled to H_2 evolution. They concluded that the reaction of *C. pasteurianum* nitrogenase has a constant stoichiometry of 2 ATP hydrolyzed per 2 electrons transferred. However, other workers have applied the same correction for reductant-independent ATP hydrolysis and have found considerably higher values[6,8]. With unfractionated nitrogenase preparations this correction is justified, because one cannot distinguish between reductant-independent ATP hydrolysis catalyzed by nitrogenase

and activity of contaminating ATPase. Our purified Mo–Fe protein and Fe protein individually contain no ATPase activity, and the small amount of reductant-independent ATP hydrolysis catalyzed by a mixture of the proteins must be attributed to nitrogenase itself. A correction for the reductant-independent activity to obtain the stoichiometry of ATP hydrolysis directly coupled to electron transfer presumes that the uncoupled ATP hydrolysis is identical in the presence and absence of reductant. That this assumption is false is shown by our observation that an excess of the Mo–Fe protein increases the ATP:$2e^-$ ratio drastically without a corresponding increase in the reductant-independent ATP hydrolysis.

Kelly[19] studied the ATP:$2e^-$ ratio with nitrogenase components from *Azotobacter chroococcum* and reported that there is an increased efficiency in use of ATP with increased amounts of the Mo–Fe protein. However, this was true only in the presence of acetylene or methyl isocyanide as reducible substrates; the effect was not observed with H_2 evolution under Ar or CO. Only two levels of proteins were used. Experiments in the presence of reducible substrates are complicated by the need for measuring both H_2 evolved and substrate reduced; it also is necessary to know the exact number of electrons involved in each reduction. Kelly reported that the ATP:$2e^-$ ratio changed when acetylene or methyl isocyanide was added to nitrogenase under Ar as compared with H_2 evolution under Ar only. This contrasts with Hadfield and Bulen's[8] observation that addition of acetylene to an Ar atmosphere does not change the stoichiometry. Our method for measuring dithionite oxidation as a measure of total electron transfer simplifies studies of the ATP:$2e^-$ ratio in the presence of various reducible substrates.

The observation that excess Mo–Fe protein inhibits electron transfer but not ATP hydrolysis suggests that at least two types of complexes between the Fe protein and the Mo–Fe protein hydrolyze ATP. One complex catalyzes ATP hydrolysis coupled to electron transfer, and another catalyzes ATP hydrolysis only. A high ratio of Mo–Fe protein to Fe protein favors the complex catalyzing uncoupled ATP hydrolysis. Each complex, however, requires the presence of reductant for maximum ATPase activity. Hadfield and Bulen[8] and Silverstein and Bulen[20] suggested that a common enzyme–ATP complex could react in two ways, both leading to ATP hydrolysis but only one providing electrons. However, if two or more kinds of enzyme–ATP complexes react with different activation energies, it may be unnecessary to postulate that the same complex reacts in two ways. An investigation of the effect of temperature at different relative levels of the Fe protein and the Mo–Fe protein should help to clarify this issue.

The lowest ATP:$2e^-$ ratio obtained in the present work was around 4. Because this appears wasteful of energy, the question arises whether or not a high ATP:$2e^-$ ratio is an inherent property of the N_2-fixing system or an artifact that arises when the cells are broken and the enzyme proteins isolated. Work with growing cultures of anaerobes indicated that a high level of ATP hydrolysis is an inherent property of nitrogenase; with *C. pasteurianum* 20 moles of ATP were consumed per mole of N_2 fixed[21] and with *Klebsiella pneumoniae* 30 moles of ATP were consumed per mole of N_2 fixed[22]. In contrast, Dalton and Postgate[23] reported that the aerobic *Azotobacter chroococcum* consumed only 5 moles ATP per mole N_2 fixed.

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