Deal, W. C., Jr. (1969), *Biochemistry 8* (in press).
Deal, W. C., Jr., and Constantinides, S. M. (1967), *Federation Proc. 26*, 348.
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., and Singh, R. M. M. (1966), *Biochemistry 5*, 467.
Hess, B., Haeckel, R., and Brand, K. (1966), *Biochem. Biophys. Res. Commun. 24*, 824.
Kayne, M. S. (1966), Ph.D. Thesis, Michigan State University, East Lansing, Mich.
Kayne, F. J., and Suelter, C. H. (1965), *J. Am. Chem. Soc. 87*, 897.
Kayne, F. J., and Suelter, C. H. (1968), *Biochemistry 7*, 1678.
Krebs, H. A., and Eggleston, L. V. (1965), *Biochem. J. 94*, 3c.
Kubowitz, F., and Ott, P. (1944), *Biochem. Z. 317*, 193.
Maeba, R., and Sanwal, B. D. (1968), *J. Biol. Chem. 243*, 448.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
Melchior, J. B. (1965), *Biochemistry 4*, 1518.
Milman, L. S., and Yurowitskii, Yu. G. (1967), *Biochim. Biophys. Acta 146*, 301.
Morawiecki, A. (1960), *Biochim. Biophys. Acta 44*, 604.
Steinmetz, M. A., and Deal, W. C., Jr. (1966), *Biochemistry 5*, 1399.
Suelter, C. H., Singleton, R., Jr., Kayne, F. J., Arrington, S., Glass, J., and Mildvan, A. S. (1966), *Biochemistry 5*, 131.
Tanaka, T., Harano, Y., Sue, F., and Morimura, H. (1967), *J. Biochem. (Tokyo) 62*, 71.
Taylor, C. B., and Bailey, E. (1967), *Biochem. J. 102*, 32c.
Tietz, A., and Ochoa, S. (1958), *Arch. Biochem. Biophys. 78*, 477.
Weber, G., Lea, M. A., and Stamm, N. B. (1967), *Life Sci. 6*, 2441.
Weber, G., Singlhal, R. L., Stamm, N. B., Lea, M. A., and Fisher, E. A. (1966), *Advan. Enzyme Reg. 4*, 59.
Weber, G., Stamm, N. B., and Fisher, E. A. (1965), *Science 149*, 65.
Yang, S. T., and Deal, W. C., Jr. (1969), *Biochemistry 8* (in press).

The Hydrolysis of Adenosine Triphosphate by Purified Components of Nitrogenase*

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ABSTRACT: The hydrolysis of adenosine triphosphate requires both purified components of the N_2-reducing system (nitrogenase): molybdoferredoxin and azoferredoxin. At low pH, the high rate of hydrolysis is not affected by the electron donor dithionite.

This shows that adenosine triphosphate hydrolysis by nitrogenase does not require a source of electrons. Evidence is also presented to suggest that even though the N_2-reducing unit and the adenosine triphosphate hydrolyzing unit are constructed with the same two protein components, these units do not contain an identical number of component molecules.

Nitrogenase, the enzyme system responsible for the biological reduction of N_2, catalyzes a transfer of electrons which is absolutely dependent upon a concomitant breakdown of ATP to ADP and P_i (Kennedy *et al.*, 1968). Thus, in the presence of ATP, nitrogenase can transfer electrons from a suitable reductant, *e.g.*, dithionite (Bulen *et al.*, 1965), to a variety of electron acceptors including N_2 (Mortenson, 1964), $2H^+$ (Bulen *et al.*, 1965), nitrous oxide (Hardy and Knight, 1966b), acetylene (Dilworth, 1966) and homologs (Hardy and Jackson, 1967), azide (Schöllhorn and Burris, 1967), cyanide and homologs (Hardy and Jackson, 1967), and isocyanides (Kelly *et al.*, 1967). On the other hand, the dependence of ATP hydrolysis upon a source of electrons does not appear to be absolute. At high pH, nitrogenase was shown (Mortenson, 1965) to hydrolyze ATP even in the absence of a source of electrons, although at a much reduced rate. The fact that at high pH the rate of hydrolysis is much higher in the presence of an appropriate electron donor has led to the assumption that in N_2 reduction, nitrogenase must get reduced before it can react with ATP. Clearly, if we are to unravel eventually the mechanism of N_2 reduction, it is important to know whether nitrogenase reacts first with the electron donor or with ATP. We therefore reexamined the hydrolysis of ATP in the presence or absence of an appropriate electron donor, using purified components of nitrogenase. We found that at low pH, the rate of ATP hydrolysis was unaffected by the electron donor dithionite. This suggests (1) that the reduction of nitrogenase is not a prerequisite for its reaction with ATP, and (2) that in N_2 reduction, the reaction of nitrogenase with ATP in fact precedes its reaction with the electron donor. These conclusions may make it easier to investigate the role of ATP in N_2 reduction. Earlier attempts to detect exchange reactions of nitrogenase involving

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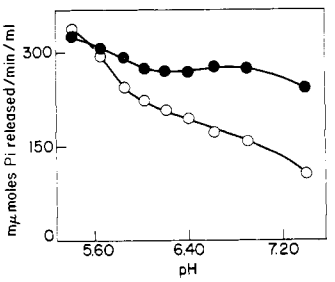


FIGURE 1: The effect of pH on the rate of ATP hydrolysis. Assay conditions are as described in Materials and Methods. (●—●) The reaction mixture also contained dithionite.

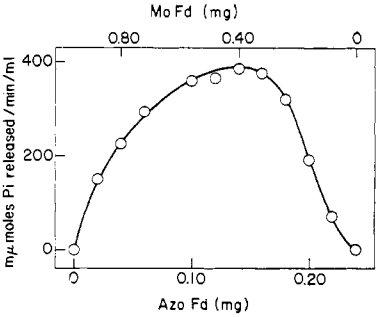


FIGURE 2: Protein requirements for ATP hydrolysis. Assay conditions are as described in Materials and Methods except that the amounts of MoFd and AzoFd were varied as shown. The initial pH of the reaction mixture was 5.4.

ATP (Bui and Mortenson, 1967; Bui, 1968) were inconclusive (Bui, 1968) partly because it had not been established whether or not dithionite was an absolute requirement for ATP hydrolysis.

Materials and Methods

Cacodylic acid (50 mM), neutralized with LiOH to give the appropriate pH, was used throughout this work as the buffering agent. Sodium salts of ATP and ADP were purchased from Calbiochem. MoFd,[1] purified to a constant Mo:Fe: protein by a modification (J. A. Morris and L. E. Mortenson, manuscript in preparation) of the method of Mortenson *et al.* (1967), was a gift from J. A. Morris. AzoFd was purified according to Moustafa and Mortenson (1969). Protein was determined according to Gornall *et al.* (1949). The assay for ATP hydrolysis was carried out anaerobically at 22° in 8-ml glass vials sealed with rubber serum stoppers. Unless indicated otherwise, the buffered reaction mixture contained in 1.0-ml total volume 2.0 mM ATP, 2.0 mM $MgCl_2$, 0.16 mg of MoFd, and 0.08 mg of AzoFd. Since the optimal concentration of $MgCl_2$, which is an absolute requirement for ATP hydrolysis, was approximately 2.0 mM for 2.0 mM ATP, the ratio of Mg^{2+} to ATP was maintained at 1.0 for all ATP concentrations. Dithionite ($Na_2S_2O_4$), when present, was at the optimal concentration of 0.5 mM. Since dithionite and the protein fractions are readily inactivated by air, they were transferred by gas-tight syringes to the vials after the vials had been thoroughly evacuated and their gas phase replaced by hydrogen. The reaction was started by the addition of ATP and stopped after 2 min, when the rate of hydrolysis is still linear, by the addition of 1.0 ml of 1.5 M trichloroacetic acid. After centrifugation, the supernatant was assayed for P_i according to Taussky and Shorr (1953).

Results

The Effect of pH on ATP Hydrolysis by Purified Components of Nitrogenase. In order to optimize the conditions for ATP hydrolysis by MoFd and AzoFd, the effect of pH was examined. The results in Figure 1 show that at high pH, the relatively slow rate of ATP hydrolysis is greatly stimulated by dithionite, but at low pH, the rate of hydrolysis is high whether dithionite is present or not. Many workers have observed the stimulation of ATP hydrolysis by dithionite at high pH. This stimulation has been interpreted to mean that nitrogenase must get reduced before it can react with ATP (see review by Hardy and Burns, 1968). However, this interpretation is not supported by experimental evidence. First, purified nitrogenase components hydrolyze ATP even in the absence of a source of electrons. Second, the presence of dithionite does not affect the rate of ATP hydrolysis at low pH. And, finally, no requirement for an electron donor could be demonstrated for the binding of MgATP or ADP to AzoFd at high pH (Bui and Mortenson, 1968). A simple alternative explanation, based on solid experimental support, for the effect of pH and dithionite on the rate of ATP hydrolysis is not available at present.

Protein Requirements for ATP hydrolysis. ATP hydrolysis at higher pH has been shown to require both protein components of nitrogenase, MoFd and AzoFd, either in the presence (Mortenson, 1965; Bulen and LeComte, 1966; Kennedy *et al.*, 1968) or absence (I. R. Kennedy and L. E. Mortenson, unpublished results) of dithionite. Figure 2 shows that at lower pH, ATP hydrolysis also requires both MoFd and AzoFd. Furthermore, the sigmoidal increase in activity with increasing concentrations of MoFd suggests that solubilized AzoFd and MoFd associate reversibly *in vitro* to form the ATP-hydrolyzing unit, AzoFd + *n*(MoFd) $\rightleftarrows$ AzoFd. (MoFd), where *n*, the minimum number of MoFd molecules, is 2.

There is evidence suggesting that even though the N_2-reducing unit and the ATP-hydrolyzing unit are constructed with the same two protein components, these units do not contain an identical number of component molecules. Mortenson (1964) observed that the minimum concentration of cell-free extract required for N_2 reduction was higher than that required for ATP hydrolysis. We interpret the results to mean that the number of component molecules required to assemble the N_2-reducing unit is larger than the number required to assemble the ATP-hydrolyzing unit. Moustafa and Mortenson (1968) have observed that in the presence of excess MoFd, the increase in acetylene-reducing activity with increasing concentrations of AzoFd is sigmoidal. This suggests to us that although only one AzoFd molecule is required to assemble the ATP-hydrolyzing unit (Figure 2), more than one AzoFd molecule is required to assemble the acetylene-reducing unit.

[1] Abbreviations used in this paper that are not defined in *Biochemistry 5*, 1445 (1966), are: MoFd, molybdoferredoxin; AzoFd, azoferredoxin.

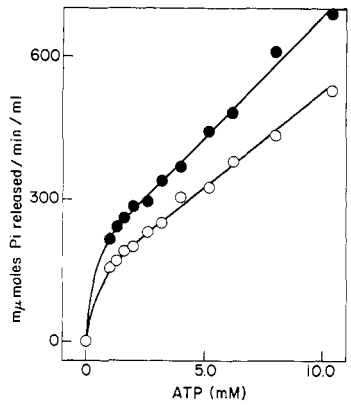


FIGURE 3: The effect of ATP concentration on the rate of ATP hydrolysis. Assay conditions are as described in Materials and Methods except that the concentration of ATP was varied as shown. The initial pH of the reaction mixture was 6.4. (●—●) the reaction mixtures also contained dithionite.

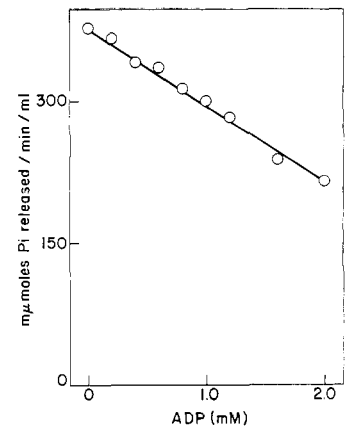


FIGURE 5: ADP inhibition of ATP hydrolysis. Assay conditions are as described in Materials and Methods except that increasing concentrations of ADP were also present. The initial pH of the reaction mixtures was 5.4.

One possibility is that the N_2- (or acetylene-) reducing unit is made up of two or more ATP-hydrolyzing units.

The Effect of Increasing Concentrations of ATP and ADP. The rate of ATP hydrolysis increases with increasing concentrations of ATP in the manner shown in Figure 3. Clearly, two phases are distinguishable: the first rapid rise in activity is followed by a slower and linear increase with no evident saturation at 10 mM ATP. The linear portion of this biphasic response is unexpected and at present, its significance is not apparent. It will be pointed out that the nonenzymic or nonspecific hydrolysis of ATP does not contribute significantly to the results in Figure 3. For example, no P_i release could be detected at 2.0 mM ATP if either MoFd or AzoFd was omitted from the reaction mixture (Figure 2). We note that (1) the activity *vs.* ATP concentration plot is sigmoidal in acetylene reduction (Moustafa and Mortenson, 1967) but not in ATP hydrolysis (Figure 4), and (2) the inhibition *vs.* ADP concentration plot is sigmoidal in cyanide reduction (Bui and Mortenson, 1968) but not in ATP hydrolysis (Figure 5). This inhibition is not caused by the competition between ADP and ATP for Mg^{2+} (Bui, 1968; Kennedy *et al.*, 1968). Since the binding site of ATP or ADP is known to be on AzoFd (Bui and Mortenson, 1968), we interpret these results to mean that the ATP-hydrolyzing unit contains only one AzoFd molecule while the N_2- (or acetylene- or cyanide-) reducing unit contains at least two AzoFd molecules. This conclusion is consistent with the protein titration data presented in the preceding section.

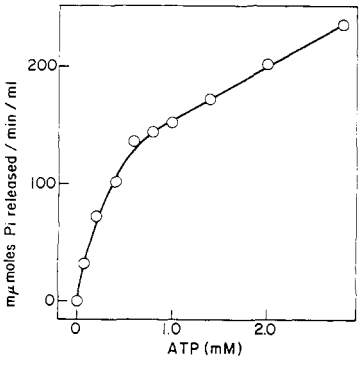
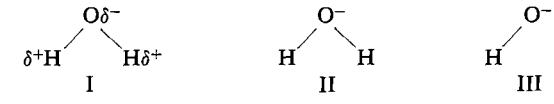


FIGURE 4: See legend for Figure 3.

Discussion

We have presented evidence to suggest that under conditions of assay *in vitro*, the hydrolysis of ATP and the reduction of N_2 (or acetylene, or cyanide) have the same protein requirements qualitatively, but not quantitatively. However, the physiological significance of these results is not clear since it is probable that in the intact organism, nitrogenase constitutes part of the cell membrane. Nitrogenase from *Azotobacter vinelandii*, for example, can be isolated as a particulate fraction which also contains protein components of the respiratory chain such as cytochromes (Bulen and LeComte, 1966). Also, the addition of lysozyme to the extracting buffer (Moustafa and Mortenson, 1969) has been found to greatly increase the efficiency of solubilizing nitrogenase from *Clostridium pasteurianum*.

The definitive explanation for the effect of pH and dithionite on the rate of ATP hydrolysis must await further work. It is tempting to offer meanwhile some speculations which, although having no experimental basis at present, should be of some value at least in explaining certain known facts in the field, and perhaps also in helping the formulation of further experiments. It is possible that the slower rate of hydrolysis at high pH, in the absence of dithionite, is caused by an inhibition by hydroxyl ions (III) which are chemically similar enough to water molecules (I) to compete for the same binding



sites. In contrast to the binding of water, the binding of hydroxyl ions leads to the formation of complexes which are unproductive because eventually, there will be no water available for the hydrolysis of ATP. Dithionite abolishes this inhibition by forming a chemical species, *e.g.*, hydrated electrons (II), capable of displacing hydroxyl ions from the water

binding sites. Since the formation of unproductive complexes with hydroxyl ions is prevented, the hydrolysis of ATP can proceed at or near the maximum rate at high pH in the presence of dithionite. If hydrated electrons are involved in N_2 reduction, their formation may require MgATP and the specific mediation by nitrogenase components since among many reductants tested, dithionite alone was capable of serving as an electron donor. Hardy and Knight (1966a) have reported that in the presence of dithionite, the increase in the number of ATP molecules hydrolyzed exactly equals the number of electrons transferred during H_2 evolution. It is intriguing that this stoichiometry is what would be expected if the proton-reducing system uses the water from each hydrated electron to hydrolyze one ATP molecule and simultaneously produces one unit of effective reducing power. The same stoichiometry has been obtained through a wide range of pH using purified MoFd and AzoFd from *C. pasteurianum* (D. Y. Jeng, J. A. Morris, and L. E. Mortenson, submitted for publication). It remains to be seen if these speculations have any basis in fact.

References

Bui, P. T. (1968), Ph.D. Thesis, Purdue University, Lafayette, Ind.

Bui, P. T., and Mortenson, L. E. (1967), *Federation Proc. 26*, 725.

Bui, P. T., and Mortenson, L. E. (1968), *Proc. Natl. Acad. Sci. U. S. 61*, 1021.

Bulen, W. A., Burns, R. C., and LeComte, J. R. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 532.

Bulen, W. A., and LeComte, J. R. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 979.

Dilworth, M. J. (1966), *Biochim. Biophys. Acta 127*, 285.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Hardy, R. W. F., and Burns, R. C. (1968), *Ann. Rev. Biochem. 37*, 331.

Hardy, R. W. F., and Jackson, E. K. (1967), *Federation Proc. 26*, 725.

Hardy, R. W. F., and Knight, E. (1966a), *Biochim. Biophys. Acta 122*, 520.

Hardy, R. W. F., and Knight, E. (1966b), *Biochem. Biophys. Res. Commun. 23*, 409.

Kelly, M., Postgate, J. R., and Richards, R. L. (1967), *Biochem. J. 102*, 1c.

Kennedy, I. R., Morris, J. A., and Mortenson, L. E. (1968), *Biochim. Biophys. Acta 153*, 777.

Mortenson, L. E. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 272.

Mortenson, L. E. (1965), *in* Non-heme Iron Proteins: Role in Energy Conversion, San Pietro, A., Ed., Yellow Springs, Ohio, Antioch, p 234.

Mortenson, L. E., Morris, J. A., and Jeng, D. Y. (1967), *Biochim. Biophys. Acta 141*, 516.

Moustafa, E., and Mortenson, L. E. (1967), *Nature 216*, 1241.

Moustafa, E., and Mortenson, L. E. (1968), *Anal. Biochem. 24*, 226.

Moustafa, E., and Mortenson, L. E. (1969), *Biochim. Biophys. Acta 172*, 106.

Schöllhorn, R., and Burris, R. H. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1317.

Taussky, H. H., and Shorr, E. (1953), *J. Biol. Chem. 202*, 675.