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NITROGENASE FROM *CLOSTRIDIUM PASTEURIANUM*

CHANGES IN OPTICAL ABSORPTION SPECTRA DURING ELECTRON TRANSFER AND EFFECTS OF ATP, INHIBITORS AND ALTERNATIVE SUBSTRATES

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SUMMARY

The iron protein of nitrogenase (Fe protein) was oxidized without inactivation in a system containing Mg·ATP and a low level of the molybdenum–iron protein of nitrogenase (Mo–Fe protein), and the optical absorption spectra of oxidized and reduced Fe protein were recorded. Oxidation increased the absorbance with the appearance of a peak at 375 nm; the spectra resembled those of bacterial type ferredoxins. A kinetic study showed that the Fe protein was oxidized at a rate that was of the same magnitude as the steady-state rate of electron transfer.

The kinetics of ATP utilization in the nitrogenase reaction was studied with the continuous assay for dithionite oxidation. No deviation from Michaelis–Menten kinetics was found; the Michaelis constant for Mg·ATP was 0.18 mM. ADP inhibited dithionite oxidation and induced deviations from the Michaelis–Menten equation.

Cyanide and azide inhibited dithionite oxidation, whereas rates of dithionite oxidation under N_2, Ar or H_2 were identical. The Fe protein was inactivated and bleached by cyanide.

INTRODUCTION

Recent studies of the EPR spectra of the nitrogenase proteins have provided experimental observations of electron transfer in the nitrogen-fixing system[1–3], and these results support a sequence of electron flow from an external electron donor to the iron (Fe) protein (azoferredoxin, Component II) and then to the molybdenum–iron (Mo–Fe) protein (molybdoferredoxin, Component I). The present communication reports optical absorption spectra of oxidized and reduced Fe protein, and the

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

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difference in absorbance between the oxidized and reduced states has been used to monitor the kinetics of oxidation of the Fe protein by the Mo–Fe protein.

The nature of the kinetics of ATP utilization by nitrogenase has been controversial, as some workers have reported normal Michaelis–Menten kinetics and others have reported more complicated kinetic patterns[4–12]. The rates in these studies were determined by fixed time assays, and because the assays usually were done with only one incubation time, initial rates may not always have been obtained. It is particularly important to exercise care to obtain initial velocities when the ATP generating system is omitted, because ADP is a strong inhibitor of the nitrogenase reaction[4]. We have previously described a continuous assay method for nitrogenase, based on spectrophotometric measurement of dithionite oxidation[13], and the present report concerns use of this method for the study of the kinetics of ATP utilization and ADP inhibition with purified nitrogenase from *Clostridium pasteurianum*. The effects of some alternative substrates and inhibitors of N_2 reduction on the rate of dithionite oxidation also have been examined.

MATERIALS AND METHODS

Purification of the nitrogenase proteins

The two nitrogenase proteins from *Clostridium pasteurianum* W5 were purified as described earlier[14].

Spectral measurements

Optical absorption spectra were recorded on a Cary Model 14 spectrophotometer. Kinetics of absorbance changes were measured on a Gilford Model 222 spectrophotometer equipped with a Beckman DU monochromator and a Honeywell Electronic 194 recorder. Quartz cuvets with a 10-mm light path were closed with rubber serum stoppers, and the solutions in the cuvets were made anaerobic by sparging with purified N_2 through hypodermic needles for 30 min.

Assays

The rate of dithionite oxidation was determined according to our published method[13,15]. Protein was determined with the microbiuret method[16] with bovine serum albumin as 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 Avenue, New York, N.Y.) heated to about 120 °C. The sodium dithionite used was from Hardman and Holden Ltd, Manchester, England. ATP (Sigma grade, disodium salt), ADP (Grade 1, disodium salt), phosphoenolpyruvate, pyruvate kinase (EC 2.7.1.40, Type II, crystalline) and creatine kinase (EC 2.7.3.2) were obtained from Sigma Chemical Company. Creatine phosphate was obtained from Pierce Chemical Company.

RESULTS AND DISCUSSION

Optical absorption spectra of the nitrogenase proteins

The nitrogenase proteins are iron–sulfur proteins, and solutions containing

them are brown. Optical absorption spectra of these proteins have been reported[11,17–19]. The spectra of the native proteins are rather featureless. Exposure of the Fe protein to air increases its absorbance around 400 nm (refs 16 and 11), but this is accompanied by a complete loss of enzymatic activity, and the significance of the spectrum of inactive, oxidized Fe protein is not clear.

The EPR spectrum of the reduced form of the Fe protein resembles EPR spectra of reduced ferredoxins, and this suggested that the optical absorption spectra of reduced and oxidized, but not oxygen-inactivated, Fe protein would show the difference around 400 nm that is characteristic of ferredoxins[20]. The nitrogenase reaction provided a method for oxidation without inactivation; that is, an excess of Fe protein was catalytically oxidized by a small amount of Mo–Fe protein in the presence of Mg·ATP. The upper spectrum in Fig. 1 was recorded for a sample that was prepared by this method. The spectrum of the reduced form in the presence of dithionite and Mg·ATP was first recorded. Addition of a small amount of Mo–Fe protein then initiated dithionite oxidation, and oxidation was monitored at 315 nm. When all the dithionite was oxidized, the absorbance at 430 nm was measured; absorbance increased rapidly, and the spectrum was recorded when no further change in absorbance at 430 nm was observed.

The ratio of the absorbances at 430 nm for oxidized/reduced Fe protein is 1.8. The spectrum of oxidized Fe protein has a broad peak with the maximum at 375 nm and resembles spectra of bacterial type ferredoxins; it lacks the peak at 460 nm that is characteristic of plant type ferredoxins[20].

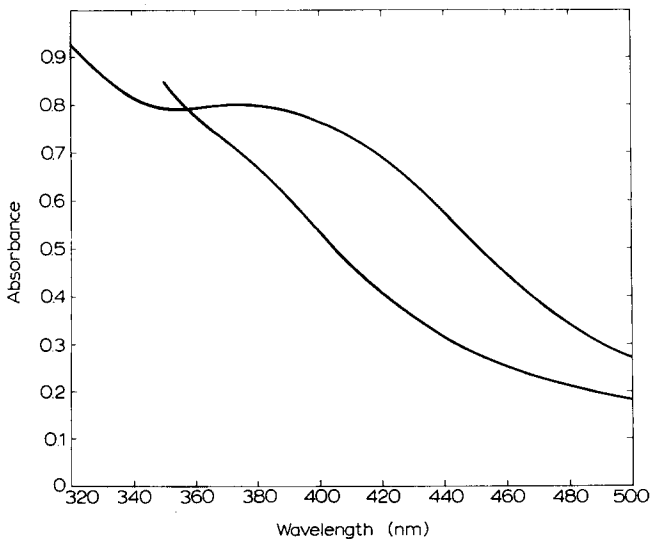

Fig. 1. Optical absorption spectra of reduced and oxidized Fe protein. Lower spectrum, reduced Fe protein: The cuvet contained 4.0 mg Fe protein/ml, 0.1 mM $Na_2S_2O_4$, 5 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 0.1 mg creatine kinase/ml and 50 mM Tris–HCl, pH 8.0 in a total volume of 1 ml. Upper spectrum, oxidized Fe protein: Same sample as for spectrum of reduced Fe protein, but 0.1 mg Mo–Fe protein/ml was added to oxidize $S_2O_4^{2-}$ and the Fe protein. The reference cuvet contained all components except Fe protein, including Mo–Fe protein for the spectrum of the oxidized protein. The spectrum of the reduced protein was not recorded below 350 nm because of the absorbance of $S_2O_4^{2-}$.

Both spectra in Fig. 1 were recorded in the presence of Mg·ATP, but spectra of reduced Fe protein in the presence or absence of Mg·ATP are identical. No method to obtain the spectrum of oxidized Fe protein in the absence of Mg·ATP has been found.

The spectral change upon catalytic oxidation of the Fe protein was reversible. The spectrum of the reduced state was observed when more dithionite was added, and subsequent catalytic oxidation led to reappearance of the spectrum of the oxidized state.

The difference in absorbance between oxidized and reduced Fe protein provides an alternative to EPR for studies of its redox properties. Kinetic studies of electron flow with the stopped-flow method should be feasible, and the absorbance change can be used for measurement of the redox potential.

Treatment of the Fe protein with Mg·ATP and small amounts of Mo–Fe protein leads to disappearance of the EPR signal due to reduced Fe protein (see Fig. 4 in ref. 1). In a preliminary experiment we have also found that the changes in EPR and optical absorbance appear to occur simultaneously. The optical absorbance change was monitored in EPR tubes in a Cary Model 14 spectrophotometer[21]. The tubes were removed quickly from the spectrophotometer at different points in the time course of oxidation and were frozen immediately by immersion in a stirred isopentane bath maintained at 130 °K; they were kept in liquid N_2 until the EPR spectra could be recorded as described in ref. 1. The decrease in EPR signal due to the Fe protein, as observed at several points in time, followed the same time course as the increase in optical absorbance.

Dalton *et al.*[18] reported that the Mo–Fe protein can be oxidized with O_2 in a reaction that is considerably faster than inactivation by O_2. This reaction is accompanied by an increase in absorbance at 435 nm. Recent EPR work[1,2], however, shows that this overoxidized state of the Mo–Fe protein does not participate in catalysis. Relative to the catalytic cycle, the native protein is in the oxidized form and can be reduced only in a system containing electron donor, Fe protein and Mg·ATP (it is not reduced directly by $Na_2S_2O_4$). These two states are spectroscopically distinguishable with EPR, and an experiment was designed to test whether the optical absorption spectra of the two states are different. The cuvet first contained dithionite and the two nitrogenase proteins at concentrations of 2 mg/ml each. After recording the spectrum, Mg·ATP, creatine phosphate and creatine kinase were added. Nitrogen fixation then started and the Mo–Fe protein was reduced, but the optical spectrum remained unchanged. Therefore, the reduction of Mo–Fe protein does not appreciably contribute to the changes in the optical absorption spectrum.

Kinetics of oxidation of the Fe protein

The absorbance increase at 430 nm during oxidation of excess Fe protein occurred rapidly, and this suggested that it would be of interest to compare the rate of oxidation of the Fe protein with the rate of electron transfer in the steady state, measured as dithionite oxidation. Time courses of absorbance changes during the steady state and during oxidation of excess Fe protein are shown in Figs 2 and 3.

The apparent Michaelis constant for dithionite in the system is very low[13]; therefore, an abrupt increase in the level of oxidized Fe protein can be observed when the dithionite becomes exhausted. The initial slow increase in absorbance shown in

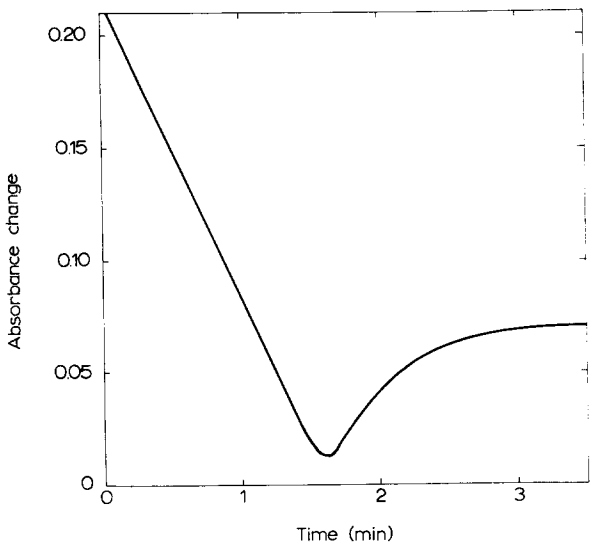

Fig. 2. Absorbance change at 315 nm during dithionite oxidation and subsequent oxidation of excess Fe protein. The cuvet contained in 1.0 ml: 2.2 mg Fe protein, 0.028 mg Mo–Fe protein, 0.1 mM $Na_2S_2O_4$, 5 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 0.1 mg creatine kinase and 50 mM Tris–HCl, pH 8.0. Temperature was 25 °C.

Fig. 3 is probably due to an increase in the steady-state level of oxidized Fe protein as the dithionite approaches exhaustion. The final phase of the curve in Fig. 2 is due to the absorbance increase at 315 nm upon oxidation of excess Fe protein; the absorbance increase is approximately 33% of the increase at 430 nm.

Dithionite acts as a two-electron donor in the nitrogenase reaction[15] and has an extinction coefficient of 8000 $M^{-1} \cdot cm^{-1}$ at 315 nm (ref. 22). The rate of electron transfer in the steady state, calculated from data in Fig. 2, is 16.5 nmoles of electrons transferred per min.

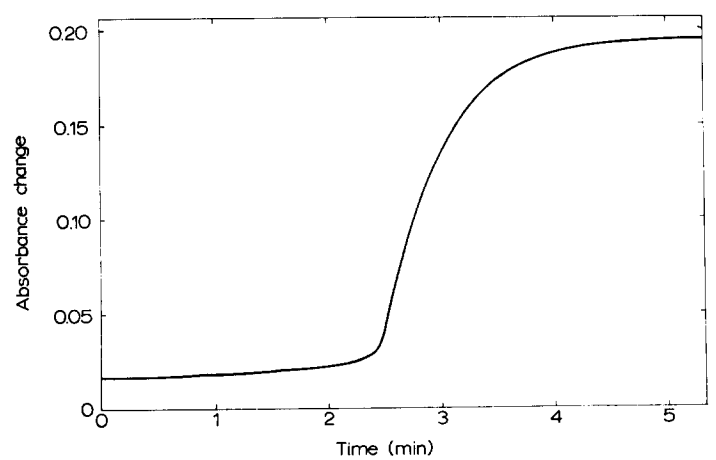

Fig. 3. Absorbance change at 430 nm during oxidation of excess Fe protein. Conditions were as described in legend to Fig. 2.

The extinction coefficient for the difference in absorbance at 430 nm between oxidized and reduced Fe protein is 4500 $M^{-1} \cdot cm^{-1}$, based upon data from Fig. 3 and a mol. wt[23] of 55 000. If each protein molecule transfers one electron[1], the maximal slope of Fig. 3 gives a rate of 27 nmoles of electrons per min, and higher numbers for electrons transferred per protein molecule would give multiples of this value. It is obvious that the rate of oxidation of the Fe protein cannot be higher than the rate of electron transfer in the steady state. A probable explanation is that the value for the extinction coefficient is too low, as the Fe protein is extremely labile, and the preparation used may have contained some inactive protein. It is therefore difficult to determine the true extinction coefficient for the Fe protein, but it seems likely that it is in the same range as for bacterial ferredoxins. The corresponding extinction coefficient for clostridial ferredoxin is approximately 7000 $M^{-1} \cdot cm^{-1}$ per one electron transfer (calculated from the extinction coefficient of oxidized ferredoxin in ref. 24 and the 55% decrease in absorbance upon reduction with dithionite indicated in ref. 20). With this value of 7000 $M^{-1} \cdot cm^{-1}$ for the extinction coefficient per one electron transfer, the calculated rates of electron transfer during the steady state and during oxidation of excess Fe protein are quite close, 16.5 *versus* 17 nmoles electrons transferred per min. The evidence presented here shows that rates of electron transfer during the steady state and during reoxidation of Fe protein are of the same magnitude; definite proof that they are equal must await determination of the exact extinction coefficient per electron transferred by the Fe protein.

It should be mentioned that the absorbance of Fe protein at 430 nm does not increase upon addition of Mo–Fe protein as long as an excess of dithionite is present.

Rates of absorbance increase at 430 nm were measured with different levels of the nitrogenase proteins. Conditions were otherwise as described in the legend to Fig. 2. With 0.028 mg of Mo–Fe protein/ml and 1.1, 2.2 or 4.4 mg of Fe protein/ml, rates were identical, but the rate with 0.014 mg of Mo–Fe protein/ml and 2.2 mg of Fe protein/ml was half of this rate. These results indicate that with excess Fe protein, the overall rate of electron transfer is zero order with respect to the Fe protein.

A possible mechanism for the oxidation of excess Fe protein involves dissociation of the active complex between the two proteins after each cycle of electron transfer is completed and subsequent combination of the Mo–Fe protein with molecules of reduced Fe protein that are free in solution. It is not known whether the two nitrogenase proteins in the steady state remain together in a complex during repeated catalytic cycles or shuttle between associated and dissociated forms, but if the presented mechanism is correct, the comparison of rates in the steady state and during oxidation of excess Fe protein suggests that both formation and dissociation of the active complex of the two proteins are as fast as or faster than electron transfer. The decrease in rate of oxidation of Fe protein with time (Fig. 3) could then be attributed to competition between oxidized and reduced Fe protein for combination with the Mo–Fe protein. Interaction among the components can be considered as an enzyme–substrate reaction with Mo–Fe protein being the enzyme and reduced Fe protein the substrate. Formation of a complex between oxidized Fe protein and Mo–Fe protein is then analogous to product inhibition. Analysis of the data in Fig. 3 shows that the rate of oxidation decreases somewhat less when oxidized Fe protein is formed than would be expected if the Mo–Fe protein showed equal preference for combination with oxidized or reduced Fe protein.

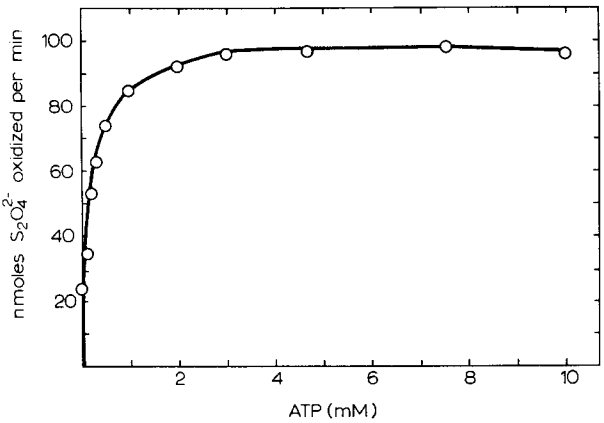

Fig. 4. Effect of ATP concentrations on the rates of dithionite oxidation. The rates were measured as described in Materials and Methods. The 1-ml reaction mixtures contained: 20 mM *N*,*N*-bis-(2-hydroxyethyl)-2 aminoethanesulfonic acid (BES) buffer, pH 7.0, 5 mM phosphoenolpyruvate, 0.1 mg pyruvate kinase, 0.3 mM $Na_2S_2O_4$, 0.60 mg Fe protein, 0.21 mg Mo–Fe protein, ATP (adjusted to pH 7.0 with 1 M KOH) as described in the figure and $MgCl_2$ to give a free Mg^{2+} concentration of 1 mM. Temperature was 25 °C.

An alternative interpretation of the observations on oxidation of excess Fe protein could be that there is exchange of electrons between different molecules of Fe protein, and that Fe protein molecules that are bound to the Mo–Fe protein can accept electrons from other Fe protein molecules. A better understanding of the interactions between the Fe protein and the Mo–Fe protein will require studies of the binding equilibrium between the two proteins.

Kinetics of ATP utilization

Reported values for the Michaelis constant for ATP in the nitrogenase reaction are in the range of 0.1–0.3 mM (refs 6, 9 and 11), but some workers have reported deviations from the Michaelis–Menten equation[4,7,8,12]. The kinetics of ATP utilization therefore were reevaluated with the continuous assay method for dithionite oxidation. The concentration of free Mg^{2+} was maintained at 1 mM (ref. 25). Fig. 4 shows a hyperbolic curve for rate *versus* ATP concentration, and the double reciprocal plot

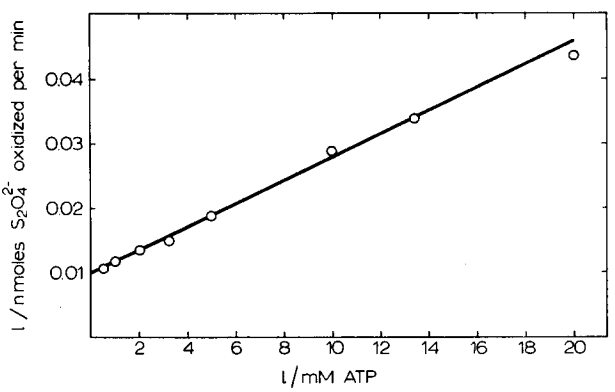

Fig. 5. Double-reciprocal plot of data from Fig. 4.

(Fig. 5) is linear; it indicates a Michaelis constant for ATP of 0.18 mM. There was no indication of sigmoidal kinetics. Another experiment showed some substrate inhibition above 10 mM ATP; at 30 mM ATP the rate was about 60% of the rate at 10 mM ATP.

Inhibition by ADP

We have shown previously[15] that rates of dithionite oxidation, determined in the absence of an ATP generating system, decrease rapidly with time. Because fixed time assays have been used in all previous studies of inhibition of the nitrogenase reaction by ADP, the reported quantitative evaluations of the inhibition are questionable. It is difficult to obtain initial rates at lower ATP concentrations even with a continuous assay method because the initial part of the time course for dithionite oxidation is affected by introduction of small amounts of O_2 during injection of the enzyme solution. Instead of measuring initial rates at different substrate and inhibitor levels, it proved advantageous to measure the rates from the curve for the time course of the reaction. ADP present at any point could be estimated from the amount of dithionite oxidized. The rates were calculated from tangents to the curve. An excess of Fe protein was used to maintain the ATP:$2e^-$ ratio at 4 (ref. 15).

Fig. 6 shows a double reciprocal plot of results from an experiment in which rates were calculated from tangents to the curves for absorbance change. Rates in the absence of ADP are omitted in Fig. 6 because of the difficulty in obtaining

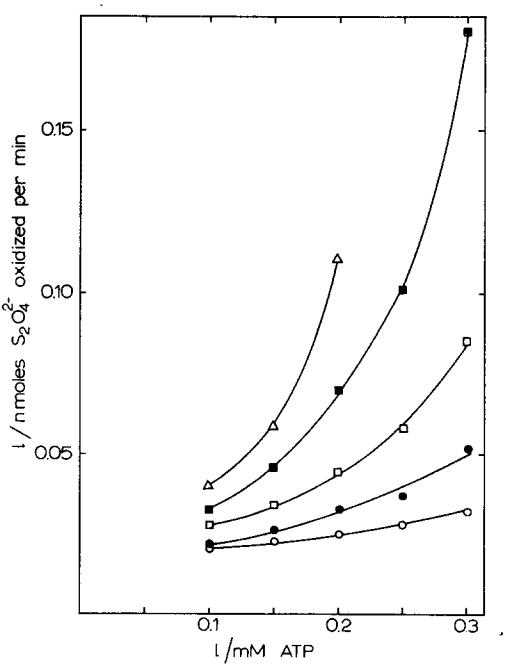

Fig. 6. Inhibition of dithionite oxidation by ADP. Rates and ADP concentrations were estimated as described in the text. The 1-ml reaction mixtures contained: 20 mM BES buffer, pH 7.0, 0.5 mM $Na_2S_2O_4$, 0.60 mg Fe protein, 0.14 mg Mo–Fe protein, ATP (adjusted to pH 7.0 with 1 mM KOH) as described in the figure and $MgCl_2$ to give a free Mg^{2+} concentration of 1 mM. Temperature was 25 °C. ○, 0.2 mM ADP; ●, 0.4 mM ADP; □, 0.6 mM ADP; ■, 0.8 mM ADP; △, 1.0 mM ADP.

accurate initial rates in the absence of an ATP-generating system. The lines curve distinctly, reflecting the response to ADP. Plots from experiments in which initial rates were determined after adding different levels of ATP and ADP, supported this conclusion, although there was more variation in the data. A control experiment with phosphoenolpyruvate and pyruvate kinase present to prevent accumulation of ADP showed that there was no decrease in the rate with time when 0.25 mM dithionite was oxidized (corresponding to formation of 1.0 mM ADP in the absence of phosphoenolpyruvate and pyruvate kinase). This proves that ADP is the inhibitor and not orthophosphate or any other product of the reaction.

The curves in Fig. 6 resemble parabolas, but no attempt has been made to fit them to any equation. Hence the curves cannot be extrapolated accurately to the y axis to determine the mode of inhibition. An experiment with higher ATP concentrations suggested that the curves intersect on the y axis (indicative of competitive inhibition), but the inhibition by high levels of ATP complicates the interpretation.

Non-hyperbolic kinetic patterns for ATP utilization in the presence of ADP have been taken as evidence that nitrogenase activity is regulated by the ratio of ATP/ADP in the cells[26], but this is not clearly established. The mechanism of ATP hydrolysis in the nitrogenase reaction is probably quite complex, and the catalytic mechanism itself may account for a complicated mode of inhibition by ADP.

Effect of some substrates and inhibitors on the rate of dithionite oxidation

Azide and cyanide are alternative substrates for nitrogenase, but their rates of reduction are lower than those for other substrates, *e.g.* N_2 or C_2H_2 (ref. 27). This could result either from increased H_2 evolution in their presence or from their inhibition of total electron flow. Electron flow can be measured as dithionite oxidation, and the results in Table I show that both cyanide and azide inhibit dithionite oxidation under Ar; cyanide is the stronger inhibitor. The table also shows nearly identical rates of dithionite oxidation under N_2, Ar and H_2; H_2 inhibits nitrogen fixation[8] but not electron flow to support H_2 evolution. The rate of total electron transfer is not dependent upon the presence of N_2. In the absence of N_2 the rate of

TABLE I

EFFECT OF SOME SUBSTRATES AND INHIBITORS ON THE RATE OF DITHIONITE OXIDATION

ATP concentration was 5 mM; conditions were otherwise as described in legend to Fig. 4. KCN and NaN_3 were used.

Gas phase	*Reducible substrates*	*Rate (nmoles $S_2O_4^{2-}$ oxidized per min)*
Ar	N_3^- (2 mM), H^+	91
		87
Ar	HCN (2 mM), H^+	40
		36
N_2	N_2, H^+	131
		126
Ar	H^+	129
		131
H_2	H^+	125
		130

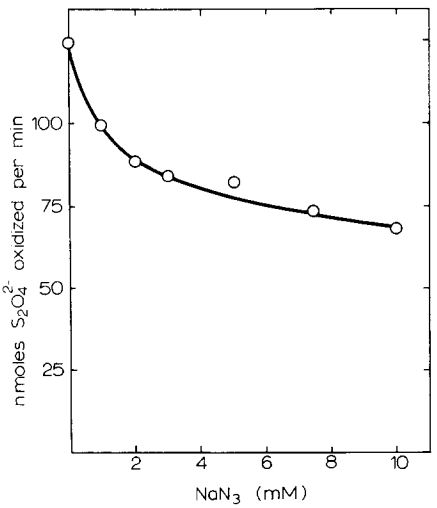

Fig. 7. Inhibition of dithionite oxidation by azide. ATP concentration was 5 mM; conditions otherwise were as described in legend to Fig. 4.

proton reduction increases sufficiently to maintain the same rate of electron transfer. Fig. 7 shows inhibition of dithionite oxidation by 1–10 mM azide; it is not a strong inhibitor of electron flow.

Inactivation of nitrogenase by cyanide

Studies of inhibition by cyanide were complicated by a rapid decrease of reaction rates with time, apparently because of irreversible inactivation of one or both of the nitrogenase proteins. These proteins are iron–sulfur proteins, and other iron–sulfur proteins are known to react with cyanide[28]. Preincubation with 10 mM KCN (neutralized) in the assay mixture showed that inactivation of the Fe protein, but not of the Mo–Fe protein, was responsible for the decrease in the rate of dithionite oxidation. When a higher concentration of Fe protein (2.5 mg/ml) was incubated at 25 °C with 10 mM KCN in 50 mM Tris–HCl at pH 8.0, a rapid bleaching of the solution was observed; most of the brown color disappeared in less than 1 h. The spectrum between 300 nm and 500 nm showed very little absorbance compared with the spectrum of untreated Fe protein (12% at 400 nm after 2 h). The time course of cyanide reduction by partially purified nitrogenase preparations previously has been reported to be linear with time[29]. The Fe protein in partially purified nitrogenase may be protected against cyanide inactivation, analogous to the protection against O_2 in particulate nitrogenase preparations from *Azotobacter vinelandii*, whereas the highly purified Fe protein from *C. pasteurianum* used in this work is sensitive to cyanide. The bleaching of the Fe protein is much faster than the bleaching of clostridial ferredoxin under similar conditions[28], and this may be related to the high sensitivity to O_2 of the Fe protein.

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