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Articles

Nitrite, a New Substrate for Nitrogenase†

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ABSTRACT: We have examined the reactivity of the purified component proteins of *Azotobacter vinelandii* nitrogenase (*Av*1 and *Av*2) toward nitrate and nitrite. Nitrate has no effect on H_2 evolution or C_2H_2 reduction by nitrogenase and thus is neither a substrate nor an inhibitor. Nitrite dramatically inhibits H_2 evolution. This inhibition has two components, one irreversible and one reversible upon addition of CO. The irreversible inhibition is due to nitrite inactivation of the Fe protein. The rate of this inactivation is greatly enhanced by addition of MgATP, suggesting the [4Fe-4S] cluster is the site of nitrite attack. The reversible inhibition does not represent an inhibition of electron flow but rather a diversion of electrons away from H_2 evolution and into the six-electron reduction of nitrite to ammonia. Thus, nitrogenase functions as a nitrite reductase.

Nitrogenase is composed of two easily separated component proteins, called the molybdenum–iron protein (MoFe protein) and the iron protein (Fe protein). The physical properties of these two proteins have been recently reviewed (Orme-Johnson, 1985), and great similarity among proteins from different bacterial sources is evident (Emerich & Burris, 1976a,b). Nitrogen fixation and all other reductions catalyzed by nitrogenase require both component proteins, a source of reducing equivalents, MgATP, protons, and an anaerobic environment (Bulen & LeComte, 1966). The MoFe protein is believed to contain the site of substrate reduction (Shah et al., 1973; Hageman & Burris, 1978; Hawkes et al., 1984), while the Fe protein is generally accepted as the specific one-electron donor for the MoFe protein (Hageman & Burris, 1978a,b; Thorneley & Lowe, 1985). In addition to N_2, nitrogenase catalyzes the reduction of protons and a number of apparently nonphysiological substrates, many of which contain triple bonds (Burgess, 1985). Such alternative substrates have been studied as probes for the number and nature of sites of substrate interaction on nitrogenase and the types of intermediates that might be formed during N_2 reduction. This paper will describe recent studies on the reactivity of nitrogenase toward two N–O bond

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containing molecules, nitrate and nitrite.

Materials and Methods

Reagents and Chemicals. ATP,[1] creatine phosphokinase, creatine phosphate, Tes, NaCl, iodine (crystalline), soluble potato starch, $NaNO_2$, and $NaNO_3$ were from Sigma Chemical Co. The specialty gases 99.997% Ar, 99.997% CO, and 1.05% H_2 in Ar (gravimetric standard) and 10% C_2H_2 in Ar were provided by Liquid Carbonic. Purified NO was kindly provided by Dr. William Evans from the UC Irvine Chemistry Department. The 1000 ppm C_2H_4 standard was provided by Alltech. $Na_2S_2O_4$ was from E-M Chemicals (a division of E. Merck).

Nitrogenase Assay. Azotobacter vinelandii MoFe and Fe proteins, designated *Av*1 and *Av*2, respectively, were purified and analyzed as described elsewhere (Burgess et al., 1980). Specific activities of the proteins were ca. 1800 nmol of H_2 min^{-1} (mg of *Av*2)$^{-1}$ and 2800 nmol of H_2 min^{-1} (mg of *Av*1)$^{-1}$. Unless otherwise indicated, all assays were performed at 30 °C in 9.5-mL calibrated vials, fitted with butyl rubber serum caps, containing the appropriate gas mixture. The 1.0-mL reaction mixture contained 38 mM Tes–KOH (pH 7.4), 2.5 mM ATP, 5.0 mM $MgCl_2$, 30 mM creatine phosphate, 20 mM neutralized $Na_2S_2O_4$ (except for dithionite utilization studies see below), and 2.5 units of creatine phosphokinase. The vessel containing the reaction mixture was degassed and filled with the appropriate gas mixture by using a published procedure (Corbin, 1978), $Na_2S_2O_4$ was added, *Av*1 was added, and the mixture was incubated at 30 °C for 5 min. Then, $NaNO_3$, $NaNO_2$, or NaCl was added when appropriate, and the reaction was started 10 s later by adding *Av*2 to give 1 mg/mL total protein at the *Av*2/*Av*1 molar ratio of 5. For assays involving salt addition, a 1.2 M stock solution in 0.1 M Tes–KOH, pH 7.4, was used. Because nitrite causes irreversible inactivation of the Fe protein (see Results), the reaction is not linear with time. Consequently, assays were run for the shortest practical time period for the measurements in question. The exact times for each assay are found in the figure legends. The assays were run with shaking at 30 °C and were quenched at the appropriate time with 0.4 M EDTA, pH 7.4 (0.2 mL), unless dithionite was to be determined (see below).

Product Analysis. With the exception of dithionite utilization studies (see below), all products were measured on the same reaction vial. All data represent means of, typically, triplicate determinations with standard deviations less than 10%. Gas samples, 200 μL at bottle pressure, were taken with a pressure-lock syringe (Precision Sampling, Baton Rouge, LA) and were analyzed with a Varian 3700 gas chromatograph with a Poropack N columm (He) and a flame ionization detector for C_2H_4 analysis and a thermal conductivity detector with a molecular sieve 13X column (Ar) for H_2 and NO analysis. Ammonia was determined by an HPLC fluorescence method described elsewhere (Corbin, 1984). Data calculations for H_2, NH_3, and C_2H_4 were performed as described previously (Rubinson et al., 1985; Robinson et al., 1986).

For dithionite utilization experiments H_2 and $S_2O_4^{2-}$ utilization were measured on the same reaction vial. These assay vials contained 3 mM $Na_2S_2O_4$. The assays were performed and data analyzed as described by Li et al. (1982) with the following modifications. The reaction vials were quenched by rapid chilling in an ice–ethanol bath. This procedure was added because the dithionite–HCHO adduct is somewhat unstable toward oxidation by NO_2^-. (Dithionite itself was insensitive to oxidation by NO_2^- over the time course of our assays.) Shortly after chilling, 0.1 mL of 40% HCHO in water was added to the vials followed by 0.1 mL of a 2% soluble potato starch solution and 0.1 mL of a 2.0 M sodium acetate solution in 0.1 M Tris, pH 7.4. This procedure differs from that of Li et al. (1982), which used a 2.0 M sodium acetate solution at pH 4.0. This modification was necessary to prevent the oxidation of the dithionite–HCHO adduct by HNO_2, which is a much stronger oxidizing agent than NO_2^-.

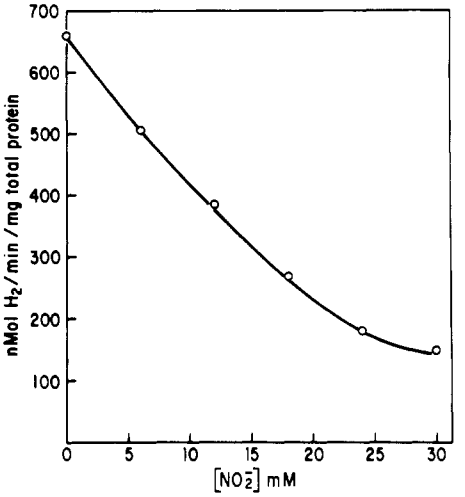

FIGURE 1: Nitrite inhibits H_2 evolution by nitrogenase. Plot of nmol of H_2 formed min^{-1} (mg of total protein)$^{-1}$ for 5-min assay under Ar. All assays contained 1 mg/mL total protein at an *Av*2/*Av*1 molar ratio of 5. For all data points, total salt concentration was adjusted to 30 mM by addition of NaCl.

Results

Reactivity of Nitrogenase toward Nitrate. H_2 evolution and C_2H_2 reduction by nitrogenase were monitored over the concentration range 0–30 mM nitrate. Total salt concentration was kept at 30 mM for all vials by addition of Cl^-. Both activities were constant regardless of NO_3^- concentration, giving values of 531 ± 12 nmol of H_2 min^{-1} (mg of total protein)$^{-1}$ and 433 ± 19 nmol of C_2H_2 min^{-1} (mg of total protein)$^{-1}$. Thus, the N–O bond containing molecule NO_3^- does not appear to be either a substrate or an inhibitor of the enzyme.

Nitrite Is an Inhibitor. Figure 1 demonstrates that, in the same concentration range where nitrate has no effect on nitrogenase, nitrite is an inhibitor of H_2 evolution under Ar.

Nitrite Inhibition Has both CO-Irreversible and CO-Reversible Components. The nitrite inhibition shown in Figure 1 could have been due to (a) irreversible inactivation of *Av*1, *Av*2, or some other component of the assay system, (b) reversible inhibition of the total electron flow of the type caused by CN^- (Li et al., 1982) or CH_3NC (Rubinson et al., 1983), or (c) a redirection of electrons away from H_2 evolution and into some NO_2^- reduction product that was not being measured. CO is known to relieve CN^--type inhibition and to inhibit the reduction of all known nitrogenase substrates except protons (Burgess, 1985). Thus, in order to distinguish irreversible from reversible inhibition, we have monitored the effects of CO on this NO_2^- inhibition.

Figure 2, panel A, shows that 60 mM NO_2^- causes ~85% inhibition of H_2 evolution under an atmosphere of 100% Ar but only 45% inhibition under an atmosphere of 5% CO/95%

[1] Abbreviations: ATP, adenosine 5′-triphosphate; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; HPLC, high-performance liquid chromatography.

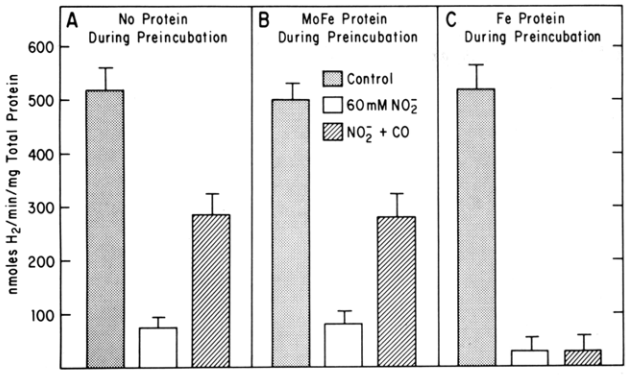

FIGURE 2: Nitrite inhibition has both irreversible and reversible components. Control vials show nmol of H_2 min^{-1} (mg of total protein)$^{-1}$ under an atmosphere of 100% Ar and 5% CO/95% Ar (three vials each) for a 2-min assay with 60 mM NaCl present during both the assay and a 5-min preincubation period. Open boxes are identical conditions except that 60 mM $NaNO_2$ replaces NaCl and the atmosphere throughout is 100% Ar. The diagonally striped boxes are identical with open boxes except that the atmosphere throughout is 5% CO/95% Ar. Preincubations were as described under Materials and Methods except that the salt was added at the beginning of the preincubation period and the proteins were added as follows. In panel A neither component protein was present during preincubation, and the assay was started by adding both components. In panel B only the *Av*1 was present, and during preincubation the assay was started by adding *Av*2, while in panel C only *Av*2 was present during preincubation and the assay was started by adding *Av*1. All assays contained 1 mg/mL total protein at an *Av*2/*Av*1 molar ratio of 5.

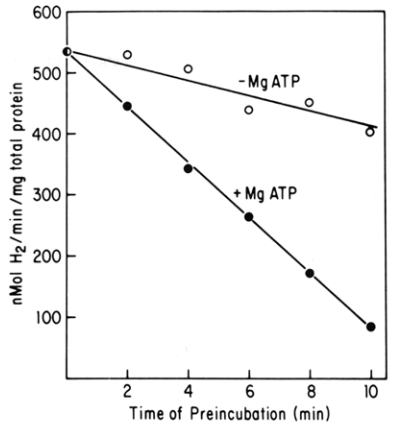

FIGURE 3: Rate of Fe protein inactivation by nitrite is enhanced by addition of MgATP. A 4.83 mg/mL Fe protein solution was preincubated for the time periods indicated with 60 mM $NaNO_2$ with (●) or without (○) 2.5 mM MgATP. At the end of the preincubation period, an aliquot of the Fe protein solution was used to initiate a standard H_2 evolution under Ar nitrogenase assay. The final [NO_2^-] in the actual assay vial was 7.2 mM, and the assay was run for 2 min. The assay contained 1 mg/mL total protein at an *Av*2/*Av*1 molar ratio of 5.

Ar. Thus, CO is able to partially protect the enzyme from NO_2^- inhibition. The simplest explanation for this result is that NO_2^- inhibition has two forms, one CO irreversible and one reversible by CO.

CO-Irreversible NO_2^- Inhibition Is Due to Inactivation of the Fe Protein. Figure 2, panel B, shows that the extent of NO_2^- inhibition under Ar and the ability of CO to protect the H_2 evolution reaction from NO_2^- inhibition are unaffected by preincubation of *Av*1 with NO_2^- relative to preincubation with no protein present (panel A). Panel C, however, shows that a 5-min preincubation of 60 mM NO_2^- with *Av*2 causes almost complete inhibition of the enzyme in the presence or absence of CO such that once the 2-min assay is started almost no product is formed. These data indicate that NO_2^- is causing CO-irreversible inhibition of the Fe protein component of nitrogenase.

For the experiments of Figure 2, MgATP was also present during the preincubation period. The binding of MgATP to the Fe protein is known to have a significant influence on the structure of the protein, apparently causing its previously protected [4Fe-4S] cluster to be exposed to the solvent (Mortenson et al., 1976; Ljones & Burris, 1978). The time dependence of the inhibition reaction shown in Figure 3 is characteristic of an irreversible inactivation reaction. (In control experiments we observed no time dependence for preincubation of the reaction mixture alone or of the MoFe protein alone.) Figure 3 shows that the rate of this Fe protein inactivation by NO_2^- is dramatically increased by having MgATP present. These data indicate that the site of NO_2^- inactivation is likely to be on the [4Fe-4S] cluster.

Inactivation of the Fe protein by NO_2^- was previously recognized by Meyer (1981), who also concluded that the [4Fe-4S] cluster was the likely site of NO_2^- attack. He suggested, however, that the inactivation of the Fe protein was not actually caused by nitrite but rather by NO which was produced nonenzymatically in the assay system. Under the conditions for the detection of H_2, our gas chromatograph registers NO eluting from the molecular sieve at 0.61 min, well separated from H_2 at 0.35 min, N_2 at 0.48 min, O_2 at 0.52 min, and CO at 0.85 min. No peaks were detected at 0.61 min during measurement of H_2 evolution for the experiments in Figures 2 and 3 or for other standard NO_2^- assays. Thus, if NO was being produced nonenzymatically in our vials, it was being produced at levels below our threshold of detectability.

Table I: Effect of Added NO on H_2 Evolution

gas-phase [NO] (atm)[a]	H_2 evolution[b]	5% CO present
0	548 ± 53	−
6.7×10^{-4}	540 ± 9	−
6.7×10^{-4}	545 ± 23	+
2.0×10^{-2}	291 ± 38	−
2.0×10^{-2}	295 ± 24	+

[a] To obtain a concentration of 6.7×10^{-4} atm, 10 μL of NO was added to a 15-mL assay vial filled with Ar. This corresponds to 30 μM NO in the gas phase or about 1.4 μM NO in the 1-mL liquid phase after correction for solubility. To obtain a concentration of 2.0×10^{-2} atm, 300 μL of NO was added to a 15-mL assay vial filled with Ar. This corresponds to 892 μM NO in the gas phase or 41 μM in the liquid phase after correction for solubility. [b] In nmol of H_2 min^{-1} (mg of total protein)$^{-1}$.

To test if such low NO concentrations could be responsible for the inhibition of H_2 evolution we observed after addition of NO_2^-, we monitored H_2 evolution by nitrogenase following the addition of easily detectable concentrations of NO. These data are shown in Table I. The NO concentration of 6.7×10^{-4} atm was 10-fold above our detection limits and was easily detected in the assay vials during H_2 evolution measurements made after the assays were run. The data in Table I show that this level of NO had no effect on H_2 evolution under Ar. We therefore conclude that the inhibition illustrated in Figures 2 and 3 could not have been caused by even lower levels of nonenzymatically produced NO and was being caused instead by NO_2^- itself. It should be noted that we did observe significant inhibition of H_2 evolution by NO at higher concentrations (Table I), which is in agreement with previous reports of NO inhibition (Rivera-Ortiz & Burris, 1975; Meyer, 1981; Trinchant & Rigaud, 1982). The inhibition of H_2 evolution observed at the higher concentrations of NO was not reversed by the addition of CO (Table I).

Nitrite Is a New Substrate for Nitrogenase: Electron Balance Studies. The CO reversible NO_2^- inhibition of H_2

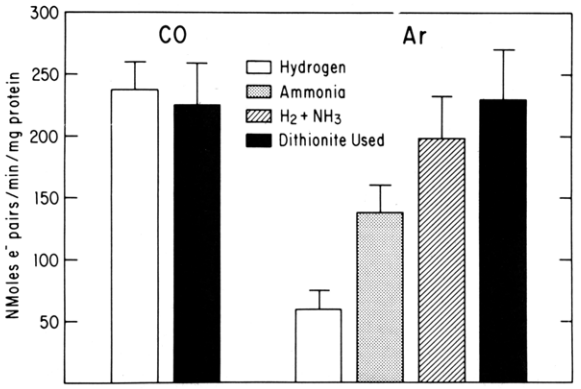

FIGURE 4: Nitrite reduction by nitrogenase. (Left) H_2 evolution and $S_2O_4^-$ utilization data for 30 mM NO_2^- under CO. Assays were run for 8 min. (Right) H_2 evolution, NH_3 production, and $S_2O_4^{2-}$ utilization data for nitrogenase in the presence of 30 mM NO_2^- under Ar. Controls without either the Fe or the MoFe protein under Ar gave no H_2 or NH_3. Controls without NO_2^- (replaced with Cl^-) under Ar also gave no NH_3. The assays contained 1 mg/mL total protein at an *Av*2/*Av*1 molar ratio of 5.

evolution shown in Figure 2 could be the result of NO_2^- inhibiting total electron flow through the enzyme in the same way that CN^- inhibits electron flow (Li et al., 1982). Alternatively, NO_2^- could be a substrate for the enzyme, redirecting electrons away from H_2 evolution and into its reduction. To distinguish these possibilities, we have directly measured the rate of electron flow by monitoring dithionite utilization. Figure 4 shows that under an atmosphere of CO with 30 mM nitrite present the rate of electron flow into nitrogenase (dithionite utilization) equals the rate of electron flow out of nitrogenase as H_2 gas. Figure 4 also demonstrates that under an atmosphere of Ar the rate of electron flow into nitrogenase in the presence of 30 mM nitrite is identical with the rate observed under CO. It is only the rate of H_2 evolution that is dramatically decreased. The finding of identical dithionite utilization rates under Ar and CO in the presence of 30 mM NO_2^- demonstrates that NO_2^- is not a CN^--type inhibitor of total electron flow.

In order to account for the difference in electron flow into nitrogenase as dithionite and out of the enzyme as H_2 gas (Figure 4, right panel), we searched for possible products of NO_2^- reduction. Figure 4 shows that ammonia is a major product of this reaction. The NH_3 is not formed in the absence of added NO_2^-, or if $S_2O_4^{2-}$, MgATP, or either of the two component proteins is left out of the assay. The formation of NH_3, like the reduction of all N_2ase substrates, is inhibited by CO (Figure 4). The reduction of NO_2^- to NH_3 requires six electron. Figure 4 shows that the sum of electron pairs that flow out of nitrogenase as H_2 and NH_3 is equal, within the limits of the measurements, to the amount of dithionite consumed. Thus, nitrogenase functions as a nitrite reductase.

Discussion

Of the N–O bond containing molecules that might be expected to react with nitrogenase only N_2O has been studied in any detail. N_2O was first implicated as a nitrogenase substrate from in vivo studies which showed that it inhibited N_2 reduction (Hoch et al., 1960; Mozen & Burris, 1954). Subsequent in vitro experiments, using *Av* and *Clostridium pasteurianum* cell-free extracts, established N_2O as an alternative nitrogenase substrate by demonstrating that the reaction required reductant and MgATP and was inhibited by CO (Lockshin & Burris, 1965; Hardy & Knight, 1966). More recently, Jensen and Burris (1986) have studied N_2O reduction using purified nitrogenase and have shown that N_2O is a competitive inhibitor of N_2 reduction and that it is not an inhibitor of electron flow. As N_2 is the major product of N_2O reduction, the reaction presumably involves cleavage of the N–O bond to yield N_2 and H_2O and suggests that the single N–O bonded resonance form $^-O–^+N{\equiv}N$ is the most appropriate representation of the substrate.

Interaction with Nitrate. The observation that nitrogenase appears to be reactive toward the N–O single bond in N_2O led us to consider the enzyme's reactivity toward the other N–O single bond containing molecules NO_3^- and NO_2^-. The interaction of purified nitrogenase with NO_3^- had not been previously investigated in spite of the fact that NO_3^- is reduced by two electrons and two protons to NO_2^- and H_2O by another (albeit dissimilar) Mo-containing enzyme, nitrate reductase. The data presented here show that NO_3^- has no effect on H_2 evolution or C_2H_2 reduction by nitrogenase and thus is neither a substrate nor an inhibitor of the enzyme.

Irreversible Inactivation of the Fe Protein by NO_2^-. The interaction of NO_2^- with nitrogenase has received more attention because this naturally occurring highly reactive anion was thought to have a possible role in vivo (Trinchant & Rigaud, 1980, 1982; Meyer, 1981). The data presented here clearly show that NO_2^- has two very different effects on nitrogenase, one CO irreversible and the other reversible by CO. The irreversible effect was previously recognized by Meyer (1981), who concluded that NO_2^- irreversibly inactivated the Fe protein component of nitrogenase probably by interaction with its [4Fe-4S] center. The data shown in Figures 2 and 3 support these conclusions of Meyer (1981) and are in conflict with the prior report by Trinchant and Rigaud (1980) that NO_2^- had no effect on the Fe protein component. Meyer attributed the irreversible effect of NO_2^- not to that anion specifically but rather to NO which was produced nonenzymatically in the assay by reaction of NO_2^- with dithionite. Because we were unable to detect NO in our assay vials (they were run for much shorter time periods) and because NO did not inhibit H_2 evolution in our assays when we added it to 10-fold above detectable levels, we conclude that NO_2^- itself is the molecule causing irreversible inactivation of the Fe protein. These data do not preclude the possibility, however, that the mechanism of inactivation involves the one-electron oxidation of the [4Fe-4S] cluster by NO_2^- to yield a nitrosyl adduct.

NO_2^- Is a New Substrate for Nitrogenase. Trinchant and Rigaud (1980, 1982) reported that NO_2^- was a reversible, competitive inhibitor of C_2H_2 reduction by partially purified *Rhizobium* nitrogenase although they concluded that it was not a substrate for the enzyme. That conclusion was based on their attempts to monitor the disappearance of NO_2^- during the course of an assay. This procedure may have been in error, however, because dithionite interferes with the NO_2^- colorimetric assay (Trinchant & Rigaud, 1982). As shown in Figure 4, we have now established that nitrite is a substrate for nitrogenase and is reduced by six electrons to ammonia. Unfortunately, the continuous inactivation of the Fe protein caused by NO_2^- during the course of the assay prevents meaningful calculation of kinetic constants for this substrate. The data in Figure 4 do suggest that NO_2^- has a fairly high K_m (on the order of 10–20 mM) when compared to other alternative substrates (Burgess, 1985). However, the pK_a of NO_2^- is ~3.8, and the possibility that HNO_2 could be the actual substrate (or irreversible inhibitor) cannot be eliminated at this time.

Nitrogenase Interaction with N–O Bond Containing Molecules: Mechanistic Considerations. In nature there are only

three known six-electron reduction reactions: the reduction of dinitrogen to ammonia catalyzed by nitrogenase, the reduction of sulfite to sulfide catalyzed by sulfite reductase, and the reduction of nitrite to ammonia catalyzed by assimilatory nitrite reductase (Guerrero et al., 1981). We now know that nitrogenase can catalyze two of these reactions. It is not known if it can also catalyze the reduction of sulfite. It is interesting to note, however, that sulfite is always present in nitrogenase assays as an oxidation product of dithionite and its reduction could pass unnoticed.

Currently there is no direct evidence to suggest how or where the N–O bond containing molecule N_2O binds to nitrogenase or to indicate the chemical mechanism of its reduction. On the basis of what is known about N_2O reduction (Jensen & Burris, 1985) and the apparently analogous reduction of N_3^- (Rubinson et al., 1985), we suggest that nitrogenase binds N_2O and catalyzes its reduction as in eq 1. By binding to the active

$$E + {}^{-}O{-}{}^{+}N{\equiv}N \rightarrow E{-}O{-}N{\equiv}N{:} \xrightarrow{2e^-,\ 2H^+} E + H_2O + N_2 \quad (1)$$

site with its anionically polarized oxygen atom, it could simply serve as a scavenger of the two electrons and two protons to form H_2O, leaving N_2 as an easily lost fragment.

The six-electron reduction of NO_2^- to NH_3 by the siroheme- and [Fe-S]-containing enzyme assimilatory nitrite reductase does not yield any partially reduced products, and the nature of nitrite binding or intermediate formation is unknown (Guerrero et al., 1981). The bent molecule NO_2^- can bind to metals in two distinct ways: the N-bound nitro form and the O-bound nitrito form (Burmeister, 1968). N-Bound forms are typically thermodynamically favored and in the present case may help explain why reduction proceeds all the way to NH_3. Electron balance studies in the present work (Figure 4) are consistent with the complete reduction of NO_2^- although they do not eliminate the possibility of small quantities of some lesser reduced product being formed. On the basis of what is known from other substrates, it is likely that the nitrogenase reaction involves a series of one- or two-electron reduction steps with enzyme-bound intermediates being formed along the way. A one-electron/two-proton reduction to give a nitrosyl intermediate is quite probably as shown in eq 2. The enzyme-

$$E \leftarrow :N(=O)O^- \xrightarrow{1e^-,\ 2H^+} E{-}N{=}O + H_2O \quad (2)$$

bound nitrosyl intermediate would then go on to be reduced by five additional electrons and five protons to yield NH_3 + H_2O. Although we have not detected NO as a product of NO_2^- reduction, future experiments will be directed toward identification of this possible enzyme-bound intermediate. If an enzyme-bound NO intermediate does occur during NO_2^- reduction, then it is probable that NO itself would be a substrate reducible to ammonia. The reactivity of nitrogenase toward NO has not been extensively studied, but it is interesting to note that like NO_2^- it is known to have both irreversible and reversible inhibitory effects (Rivera-Ortiz & Burris, 1975; Meyer, 1981; Trinchant & Rigaud, 1982).

The other N–O bond containing molecule we have considered here is NO_3^-. It is neither a substrate nor an inhibitor for the enzyme. There are two possible reasons why N_2O and NO_2^- are substrates while NO_3^- is not. First, N_2O and NO_2^- are softer, more polarizable ligands, and they may bind more readily to the active site. Second, NO_3^- is an anion of high thermodynamic stability, and there may simply be a much greater activation energy barrier to the reduction of its first nitrogen–oxygen bond.

Conclusions

In summary, our studies have shown the following: (1) NO_3^- is neither a substrate nor an inhibitor of nitrogenase, (2) NO_2^- causes irreversible inactivation of the Fe protein probably by reaction with its [4Fe-4S] cluster, and (3) NO_2^- is a new substrate for nitrogenase and is reduced by six electrons to ammonia.

Acknowledgments

We are pleased to acknowledge the talents of Charles Miller and Teresa Chun in cell growth and protein purification.

Registry No. NO_2^-, 14797-65-0; NO_3^-, 14797-55-8; NO, 10102-43-9; nitrogenase, 9013-04-1.

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Purification and Characterization of an Isoform of Protein Kinase C from Bovine Neutrophils†

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ABSTRACT: Protein kinase C (PKC) from bovine neutrophils was purified 1420-fold. Subcellular fractionation analysis of bovine neutrophil homogenate in the presence of EGTA indicated that more than 95% of the PKC activity was present in the soluble fraction. The purification procedure from cytosol involved sequential chromatographic steps on DE-52 cellulose, Mono Q, and phenyl-Sepharose. Whereas bovine brain PKC could be resolved into four isoenzymatic forms by chromatography on a hydroxylapatite column, bovine neutrophil PKC was eluted in a single peak, suggesting that it corresponded to a single isoform. The apparent molecular weight of bovine neutrophil PKC was 82000, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. By filtration on Sephadex G-150, a molecular weight of 85000 was calculated, indicating that bovine neutrophil PKC in solution is monomeric. Its isoelectric point was 5.9 ± 0.1. Bovine neutrophil PKC was autophosphorylated in the presence of [γ-^{32}P]ATP, provided that the medium was supplemented with Mg^{2+}, Ca^{2+}, phosphatidylserine, and diacylglycerol; phorbol myristate acetate could substitute for diacylglycerol. Autophosphorylated PKC could be cleaved by trypsin to generate two radiolabeled peptides of M_r 48000 and 39000. The labeled amino acids were serine and threonine. During the course of the purification procedure of bovine neutrophil PKC, a protein of M_r 23000, which was abundant in the cytosolic fraction of the homogenate, was found to exhibit a strong propensity to PKC-dependent phosphorylation in the presence of [γ-^{32}P]ATP, Mg^{2+}, Ca^{2+}, phosphatidylserine, and diacylglycerol. This protein was recovered together with PKC in one of the two active peaks eluted from the Mono Q column at the second step of PKC purification. It is suggested that the M_r 23000 protein might be a natural substrate for bovine neutrophil PKC.

Protein kinase C, a Ca^{2+}- and phospholipid-dependent kinase (PKC)[1] of $M_r \simeq 80000$, has been found in a large number of tissues and appears to play a central role in cellular economy [for review, see Nishizuka (1984)]. Recently, the existence of a family of PKC genes has been reported (Knopf et al., 1986; Parker et al., 1986; Coussens et al., 1986; Ono et al., 1986; Ohno et al., 1987). Three cDNA clones were obtained from rat, bovine, human, and rabbit libraries, and two sequences from rat brain were shown to derive from alternative splicing of a single clone (Kubo et al., 1987). Isoforms of PKC have been identified after separation on a hydroxylapatite (HTP) column from rat brain (Huang et al., 1986a; Kikkawa et al., 1987) and rabbit brain (Jaken & Kiley, 1987). PKC isoforms have also been reported in rat liver (Azhar et al., 1987), in murine fibroblasts (McCaffrey et al., 1987), in rat spleen (Brandt et al., 1987), and in bovine adrenocortical tissue (Pelosin et al., 1987).

It is a current view that PKC in neutrophils accomplishes a number of strategic functions, following exposure of these cells to specific stimuli or to phorbol myristate acetate (PMA), in particular the activation of the respiratory burst [for reviews, see Rossi (1986) and Tauber (1987)]. Our group is interested in the activation of the respiratory burst in bovine neutrophils (Ligeti et al., 1988). We therefore decided to explore in more detail the nature, properties, and function of PKC from bovine neutrophils. The present paper describes a simple and rapid method of purification of PKC from bovine neutrophils and some properties of this enzyme. The search for isoforms of neutrophil PKC is also reported, together with the demonstration of a PKC-dependent phosphorylation of a small mo-

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[1] Abbreviations: PKC, protein kinase C; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethane sulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PMA, 4β-phorbol 12-myristate 13-acetate; ATP, adenosine 5′-triphosphate; HTP, hydroxylapatite; M_r, molecular weight; SDS, sodium dodecyl sulfate.

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