Vol. 93, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1980 Pages 934-940

REGULATION OF NITROGENASE ACTIVITY IN AEROBES BY MG^{2+} AVAILABILITY: AN HYPOTHESIS

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Received March 5, 1980

SUMMARY

Nitrogenase functions at or near its maximum capacity *in vivo*, despite a reported energy charge in the cell that should severely inhibit the enzyme. Deenergizing cellular membranes, which is postulated to release magnesium in mitochondria, has been reported to produce rapid inhibition of nitrogenase activity while giving only small changes in energy charge and NAD^+/NADH ratio. It is proposed that the level of magnesium available for complexation by the potent inhibitor ADP is the rate controlling variable for nitrogenase activity

The enzyme nitrogenase reduces N_2, and a range of other substrates containing triple bonds, at the expense of energy from ATP and electrons. For each mole of N_2 reduced, a minimum of 12 moles of ATP is used directly, in addition to six electrons (three pairs) (1), each of which could yield an additional 3 ATP in intact *A. vinelandii*, implying a minimum energy cost of 21 ATP per mole of N_2 reduced. Nitrogenase activity requires both a MoFe protein and an Fe protein (1). When Fe protein is limiting *in vitro*, the ATP expenditure per mole of N_2 reduced may increase without limit (2). Clearly, such an enzyme must be under fine control in the cell.

Evidence presented below indicates that *in vitro* nitrogenase is more sensitive to changing ADP/ATP than would be expected if energy charge were the controlling variable. Conversely, measured energy charge values imply that nitrogenase ought to be potently inhibited *in vivo*. With 10 μmolar nitrogenase in the cell (3) and a turnover number of 13 ATP/sec (4), we estimate that nitrogenase would deplete the cell of 2 mM ATP (5)

Abbreviation:
TCA, trichloroacetic acid

0006-291X/80/070934-07$01.00/0

within <15 seconds in the absence of an energy input. This rapid ATP usage may, in turn, make energy charge measurements difficult (see below).

Evidence that nitrogenase activity is fully expressed in vivo. Nitrogenase must function near maximal capacity to allow the relatively rapid doubling times observed with Azotobacter. In a chemostat culture, at a dilution of 0.2 hr^{-1}, a culture containing 2.56 mg of bacterial protein per ml which must have reduced ~90 nmoles N_2/min, showed an in vivo acetylene reduction rate of 100 nmoles/min[1] (6). In vitro the activity was about 45 nmoles C_2H_2 reduced/min/mg protein, which may be an underestimate of 10-20% due to dilution effects (7). It appears that the nitrogenase activity is fully expressed in vivo; there is no evidence for "cryptic" nitrogenase enzyme or surplus functional capacity over the amount needed by the cells.

Nucleotide levels and energy charge in A. vinelandii. Haaker et al. (5) observed a total adenosine nucleotide content of about 15-20 nmoles/mg bacterial protein in oxygen-adapted cells. Knowles and Smith (8) found ATP levels of 5-6 nmoles/mg cell dry weight in late log phase cells of A. vinelandii. The estimated ATP concentration in A. vinelandii thus is 1-2 mM if one assumes 5-6 µl H_2O/mg protein (5) or 3 µl H_2O/mg dry weight (8).

One reasonable control mechanism would be for nitrogenase to show strong "allosteric behavior" with ADP turning it off at relatively low concentrations, i.e., at a high energy charge (E.C.). The measured value of E.C. in A. vinelandii, defined as [(ATP) + ½(ADP)]/[(ATP) + (AMP)], may be between 0.6 and 0.85 under various conditions, according to the data of

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[1] At this dilution rate, 2.56 mg protein was produced in 5 hr (300 min) corresponding to 380 µg N if we assume a typical N content of about 15% in protein (6). Hence 1.28 µg N must be fixed per min, or nearly 90 nmoles/min. This is 135 nmoles/min electron pairs to N_2, plus some to H_2, compared to the measured value of 100 nmoles/min acetylene reduced. Kleiner also reported (23) that a culture diluted at 0.24/hr showed about 70 nmoles acetylene reduced/min/mg bacterial protein. If 1 mg protein contains 150 µg N, about 0.53 µmoles of N_2 must be reduced every 250 min, or 21 nmoles/min/mg for protein synthesis alone plus additional N as needed for nucleic acids. This is close to the observed electron flow to acetylene (63 nmoles/min/mg to N_2, plus some amount of H_2, vs. 70 nmoles/min/mg to acetylene).

Haaker et al. (5) and Liao and Atkinson (9). Such levels of ADP might reduce the activity of nitrogenase without unduly inhibiting other important kinases in the cell.

Liao and Atkinson (9) studied E.C. of A. vinelandii over an entire growth cycle and found that E.C. was near 0.85 until the cells reached a high density at which time it declined to 0.6. Haaker et al. (5) grew cells to limiting oxygen levels and then varied oxygen concentration at relatively high cell densities which could have accounted for the rather low E.C. in their experiments. However, the reported acetylene reduction activity (∿50 nmoles/min/mg protein in oxygen-adapted cells) was slightly better than observed by Kleiner (6).

Sensitivity of nitrogenase to ADP inhibition. The proposal of Moustafa and Mortenson (10) that ADP might be a negative modifier of nitrogenase activity has been confirmed in recent studies both in vitro (11,12) and in vivo (5,13). Thorneley and Cornish-Bowden (12), using Klebsiella pneumoniae nitrogenase, found a K_i of 20 μM for ADP inhibition of the ATP promoted electron transfer between the Fe protein and MoFe protein of nitrogenase, and estimated K_a for ATP as 400 μM. In steady-state H_2 evolution experiments, they found that 700 μM ADP gave 50% inhibition of H_2 evolution in the presence of 5 mM ATP with 10 mM magnesium.

Table I. Effect of ADP on ATP hydrolysis and H_2 evolution by A. vinelandii nitrogenase

Initial† ADP/ATP	Initial rate (nmoles/min) P_i release	H_2 evolution
0	125	27.5
.04	90	19
.08	76	14
.125	60	11

†Assays were done as described (Sotaro Kotake, M.S. Thesis, Kansas State University, 1979). Reaction mixtures of 1.0 ml contained 20 mM dithionite, 5 mM ATP, 10 mM magnesium in pH 7.4, 25 mM K^+ HEPES buffer. ADP was added as indicated. Reactions were initiated by adding 0.1 ml enzyme (11.5 μM MoFe and 28.6 μM Fe proteins). Individual samples were stopped at intervals of from 1 to 6 min. Inorganic phosphate was determined by the method of Rathbun and Betlach (22); H_2 evolution by gas chromatography.

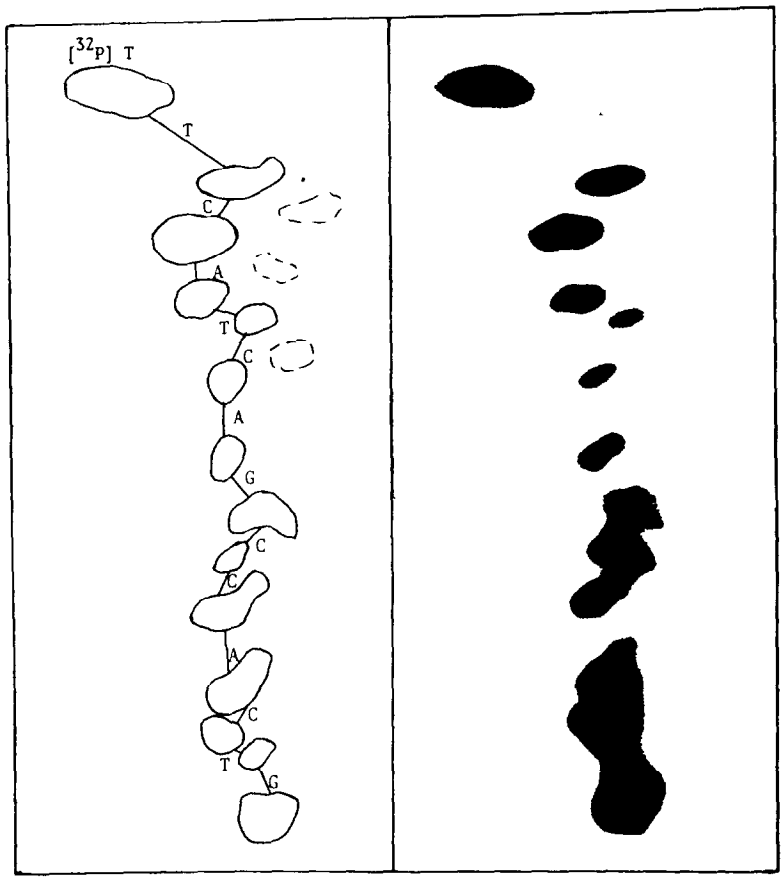

Fig. 2. Two Dimensional Mobility Shift Chromatography of the Oligonucleotide dTTCATCAGCCACTG. Conditions as described in Methods.

using two dimensional homochromatography. As shown in Fig. 1 the product oligonucleotide had the sequence dTTCATCAGCCACTG. The radioautograms were intentionally overexposed to show minor spots.

^{32}P-labeled 14 mer specifically hybridized to human islet or rabbit pancreas A^+ RNA covalently bound to paper chips. The extent of reaction was directly proportional to the amount of RNA (Fig. 2A). Control reactions utilizing yeast RNA and rat liver RNA under the same conditions yielded no detectable hybrids, indicating specific hybridization with pancreas RNA. This result was confirmed by solution hybridization between human islet RNA and ^{32}P-14 mer using gel-filtration as shown in Fig. 3B. Similar data were obtained for rabbit pancreas total RNA which bound 43 fmol 14-mer/mg RNA. We estimate the detectable level of glucagon mRNA to be less than 0.1 fmol.

1 mM. Thus Mg^{2+} potentiated the inhibiting effect of ADP on nitrogenase activity and may be a primary effector of the enzyme *in vivo*.[2] Haaker *et al.* (5) observed that the functioning of nitrogenase depended not on the ratio of NAD(P)H/NAD(P), nor simply on ADP/ATP but rather on the extent to which the membrane was energized. They suggested that electron supply from the energized membrane to nitrogenase was controlling for *in vivo* activity although the route of electron flow from membrane to the Fe protein of nitrogenase remained unspecified. Others have proposed that electrons are donated to Fe protein by a flavoprotein (14). Because the Fe protein has been shown to be a freely dissociable component of nitrogenase (15), and is present in several-fold molar excess (7), there is really no need to postulate a special electron carrier. Hageman and Burris (15) have proposed renaming the Fe protein as a nitrogenase reductase and the MoFe protein as the nitrogenase enzyme.

Postulated role of membrane energization and ion binding. There is considerable evidence that in mitochondria, energizing the membrane is related to tight binding of divalent metal ions. When the membrane is deenergized, or ATP synthesis is uncoupled, there is often an efflux of these ions (16,17,18,19).

We propose that the level of bound *vs.* free magnesium, rather than electron supply or energy charge, is the controlling variable for nitrogenase *in vivo*. If the published measurements of energy charge in *A. vinelandii* are correct (5,9) then the ratio of ADP/ATP is between 0.5 and 0.13 and nitrogenase should be significantly inhibited *in vivo* (90% by our estimation) when there is sufficient free magnesium to bind to ADP. Yet the best available evidence suggests that nitrogenase is fully functional

[2] We measured Mg^{2+} in a cell paste of *A. vinelandii* grown under N_2 fixing conditions (7) and found 545 ppm. This corresponds to about 40 mM in the cells, assuming the paste is $<\frac{1}{2}$ free water.

in vivo. Either the published energy charge levels are much too low or magnesium availability must be considered in calculating an effective energy charge for control of nitrogenase activity. If the energized call membrane binds magnesium, then nitrogenase can be fully functional in the presence of relatively high levels of ADP, but once the membrane is deenergized, nitrogenase may be rapidly inhibited by magnesium-ADP. Control of available magnesium concentration may be a powerful mechanism for amplifying the effects of changing energy charge.

There is no reason that the proposed control mechanism need be limited to *A. vinelandii*. It may pertain in all of the "aerobic" nitrogen-fixers in which ATP is synthesized by energized membrane processes. Appleby *et al.* (13) observed that there was a proportional response of nitrogenase activity of intact *Rhizobium* bacteroids with changing ADP/ATP ratio, similar to that observed *in vitro* with *C. pasteurianum* nitrogenase enzyme (11), where a ratio of ADP/ATP near 1 was needed to turn off the enzyme. The lowest (most favorable) ratio of ADP/ATP that Appleby *et al.* (13) observed was 0.2 while the highest enzyme activity observed was only 5 nmoles C_2H_2 reduced/min/mg bacteroid dry weight, which is about 10-fold less than the specific activity of *A. vinelandii*. Israel *et al.* (20) prepared the nitrogenase MoFe protein from bacteroids and observed a specific activity of 30 nmoles/min/mg protein in crude extracts. Thus the intact bacteroids studied by Appleby *et al.* (13) apparently were relatively inactive and they may not have been studied in the critical control range of the cells. Alternatively, the *Rhizobium* nitrogenase may differ from *A. vinelandii* in its reponse to energy charge. Energy charge has been estimated in intact nodules (21) and ranges from 0.9 to 0.35 depending on respiration rate. The sensitivity of *Rhizobium* nitrogenase to magnesium and ADP inhibition *in vitro* remains to be established. The mode of control of nitrogenase activity has important implications for genetic engineering experiments since nitrogenase must be functional under conditions which prevail in the recipient cell if transfer of genetic material is to be fruitful.

ACKNOWLEDGMENTS

Contribution #80-273-j, Kansas Agricultural Experiment Station.

The research described herein was supported by a grant from the National Science Foundation: PCM7815250.

We thank M. Weston for excellent technical assistance.

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