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Cellular ATP Levels and Nitrogenase Switchoff upon Oxygen Stress in Chemostat Cultures of *Azotobacter vinelandii*

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When *Azotobacter vinelandii*, growing diazotrophically in chemostat culture, was subjected to sudden increases in the ambient oxygen concentration (oxygen stress), nitrogenase activity was switched off and cellular ATP pools decreased at rates depending on the stress level. Following a fast decrease, the ATP pool approached a lower level. When the stress was released, these effects were reversed. The reversible decrease of the ATP pool upon oxygen stress could also be observed with cultures assimilating ammonium and, at the same time, fixing dinitrogen because of growth at a high C/N ratio but not with cultures growing only at the expense of ammonium. When strains OP and UW136 of *A. vinelandii* were subjected to long-term increases in ambient oxygen, the sizes of cellular ATP pools eventually started to increase to the level before stress and diazotrophic growth resumed. The cytochrome *d*-deficient mutant MK5 of *A. vinelandii*, however, impaired in aerotolerant diazotrophic growth, was unable to recover from stress on the basis of its ATP pool. The results suggest that adaptation to higher ambient oxygen depends on increased ATP synthesis requiring increased electron flow through the entire respiratory chain, which is possible only in combination with the more active, yet possibly uncoupled, branch terminated by cytochrome *d*. It is proposed that the decrease of the cellular ATP level under oxygen stress resulted from the increased energy and electron donor requirement of nitrogenase in reacting with oxygen.

The nitrogenase enzyme complex, catalyzing the reduction of dinitrogen to ammonia, is highly sensitive to oxygen. Nevertheless, the aerobic *Azotobacter vinelandii* is able to grow diazotrophically up to the highest dissolved-oxygen concentrations, about 230 μM, which can be reached with air (19). Postgate and colleagues and Yates (4, 5, 8, 26) proposed that nitrogenase is protected against the damaging effect of oxygen either by high cellular respiration or by a conformational change within the enzyme protein. According to the hypothesis of respiratory protection, oxygen is consumed already at the cell's surface so that it is prevented from entering the cell. Formulation of this hypothesis was based on the facts that azotobacters exhibit one of the highest respiratory activities among aerobic organisms and that an increased respiratory activity could be observed when cells were grown at an elevated oxygen concentration (8, 10). Increased respiration is assumed to be accompanied by uncoupling of electron transport, allowing increased oxygen consumption without increasing ATP production (9). Partial uncoupling of cellular respiration is possible because the respiratory system of azotobacters is branched with either cytochrome *o* or cytochrome *d* as terminal oxidases, with the latter branch believed to be uncoupled (9). In agreement with the proposed functions of the uncoupled branch in respiratory protection, a mutant of *A. vinelandii* defective in the formation of cytochrome *bd* was reported to be impaired in aerotolerant diazotrophic growth (11). On the other hand, a mutant which exhibited increased levels of cytochrome *bd* but no detectable amounts of cytochrome *o* was able to fix dinitrogen in air (15).

Respiratory protection is assumed to be active while cells are fixing dinitrogen in the presence of oxygen. Whenever this steady state becomes disturbed by a sudden increase in the dissolved-oxygen concentration (oxygen stress), conformational protection becomes effective and, at the same time, nitrogenase activity of azotobacters is reversibly switched off. Switchoff occurs as well if ammonium is added to diazotrophically growing cultures. This switchoff, however, was reported to be dependent on the assimilation of ammonium (3, 20). Investigations on the mechanism underlying oxygen-induced switchoff revealed that the two subunits of the nitrogenase complex form a three-component complex in combination with a low-molecular-weight (two atoms of iron and two atoms of sulfur) protein (21). Formation of this three-component complex depends on the oxidation of nitrogenase as well as of its electron donor (21, 24, 27). The three-component complex dissociates and nitrogenase activity is switched on as soon as the enzyme returns to the proper reduction state either when the oxygen stress is released or when, according to the hypothesis of respiratory protection, the cells are allowed to increase the rate of oxygen consumption by synthesizing the uncoupled branch of the respiratory system (8).

However, detailed investigations on the dependence of cellular oxygen consumption on the dissolved-oxygen concentration, performed with strictly controlled chemostat cultures of *A. vinelandii*, revealed that respiration does not increase linearly with increasing oxygen concentrations (19). Subsequently, the lack of proportionality between ambient oxygen concentrations, cellular respiratory rates, and the function of nitrogenase was confirmed by a number of independent observations (13). In this context it is interesting to note that the above-mentioned cytochrome *o*-deficient mutant of *A. vinelandii* exhibited the same oxygen tolerance as the wild type but only 45% of its respiratory activity (15). All of these observations as well as the observation that oxygen enters diazotrophic cells (18) suggest that oxygen consumption at the cell's surface is not the only mechanism protecting nitrogenase from inactivation. Since switchoff was shown to be dependent on the rate

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of substrate supply, i.e., the supply of reducing equivalents and energy, rather than on the activity of cellular oxygen consumption, we proposed that, under steady-state conditions, the flux of electrons through the enzyme complex maintaining a low redox state protects nitrogenase from switchoff (13). It has been suggested for *Azotobacter chroococcum* that, if oxygen enters the cell because of insufficient respiratory protection, reduction of oxygen by nitrogenase may help protect nitrogenase from inactivation (22). This reaction with oxygen, termed "autoprotection," implies that nitrogenase is able to reduce oxygen as long as the cells can keep the enzyme in a reduced state. Reaction of nitrogenase with oxygen creates toxic oxygen radicals, which, however, can be removed by enzymes like catalase, peroxidase, and superoxide dismutase present in nitrogen-fixing azotobacters (7, 22).

Since it is generally accepted that reduction of nitrogenase requires not only reducing equivalents but also magnesium-ATP, the question of whether there is a relationship between these factors and inactivation of nitrogenase under oxygen stress arises. In the present investigation we concentrated on the effect of oxygen stress on both the cellular ATP level and the switchoff of nitrogenase activity. On the assumption that ATP represents a substrate for nitrogenase, we chose chemostat cultures for the experiments because in this culture system there is a strict dependence of cellular activities on the steady-state level of the limiting substrate. The results reveal that oxygen stress leads to a sudden decrease of the cellular ATP level. It is proposed that nitrogenase activity is warranted as long as the regeneration of ATP is sufficiently high.

MATERIALS AND METHODS

Bacterial strains and culture conditions. If not stated otherwise, *A. vinelandii* OP (ATCC 13705) was used throughout the experiments. Mutant strain MK5 of *A. vinelandii*, lacking cytochrome *bd* (11), as well as its parent strain, UW136, were kindly provided by R. K. Poole (Kings College, London, United Kingdom).

The organisms were grown in an oxygen- and pH-controlled chemostat at a constant dilution rate of 0.15 h^{-1}. Dissolved oxygen concentrations were measured and kept constant as described previously (19). Diazotrophic cultures were grown in nitrogen-free medium (19) with 0.3% glucose as the growth-limiting substrate. In cultures grown with different molar ratios of C and N atoms, the concentration of glucose was varied while the concentration of ammonium was constant at 2.5 mM. Sodium citrate was added to a final concentration of 0.1 g/liter in order to complex iron ions. The growth medium for mutant MK5 contained kanamycin at a final concentration of 1 mg/liter. Conditions of oxygen stress were achieved as described previously (13).

Activity measurements. Nitrogenase activity was determined in situ after injection of acetylene (2% [vol/vol]) into the headspace of the fermentor (19). The specific rate of nitrogen fixation was calculated by the method of Bühler et al. (2). Respiratory activities of whole cells were measured polarographically (YSI 35; Yellow Springs Instrument Co., Yellow Springs, Ohio) after samples were suspended from chemostat cultures in air-saturated fresh medium. In situ respiratory activities were calculated on the basis of the rate of gas flow through the culture and the difference between oxygen concentrations at the gas inlet and outlet (1).

Determination of ATP. In order to quantify cellular ATP levels, culture samples (5.0 ml) were quickly withdrawn from chemostats into syringes containing 1.5 ml of precooled (0°C) extraction medium (0.027 M EDTA in 1.6 M perchloric acid) by the method of Lundin and Thore (17). This ensured rapid mixing of the culture sample with the extractant. After 20 min of incubation at 0°C the pH of extracts was adjusted to 7.75 with 1.6 M KOH containing 80 mM $KHCO_3$ and 50 mM Tris. The extracts were kept on ice for 1 h to precipitate $KClO_4$, which was subsequently removed by centrifugation (10 min at 27,000 × *g* and 4°C). The resulting supernatants were stored at −20°C before ATP measurements. After 10-fold dilution of the samples, ATP was determined by the firefly luciferase assay using Lumat LB 9501 (Berthold, Wildbad, Germany) by the method of Wulff and Döppen (25) with the following modifications. Solution 1 contained 20 mM Tris, 0.1 mM EDTA, and 5 mM magnesium acetate. Concentrations of luciferin and luciferase in the mixture injected into the sample were 714 μM and 5 mg/liter, respectively. For ATP determination 0.1 ml of the diluted sample plus 0.3 ml of solution 2 and 0.1 ml of the luciferin-luciferase mixture were thoroughly mixed. All of the enzymes and substrates were from Boehringer (Mannheim, Germany). An internal standard was used to calculate the ATP concentration of the samples. Changes in ATP levels were calculated from semilogarithmic plots of ATP values versus time.

TABLE 1. Switchoff of nitrogenase and rates at which the cellular ATP levels decreased upon oxygen stress[a]

O_2 (μM)	Switchoff (%)	ATP	
		μ_{ATP} (h^{-1})[b]	nmol h^{-1} mg^{-1}[c]
85	60	−1.43	−18.4
104	100	−2.08	−26.8
127	100	−2.34	−30.2

[a] Chemostat cultures of *A. vinelandii* growing with 68 μM O_2 under steady-state conditions at a dilution rate of 0.15 h^{-1} were subjected to oxygen stress for 7 min.
[b] Steady-state μ_{ATP}, +0.15 h^{-1}.
[c] Specific rate. Under steady-state conditions, the specific rate at which the cellular ATP pool was kept constant was +1.9 nmol h^{-1} mg^{-1}.

Quantitative determinations. Protein was determined by the method of Lowry et al. (16). Levels of glucose were determined with a test combination (Boehringer). All of the results presented in this article are based on two or three measurements per sample of two or three independent cultures.

RESULTS

Oxygen stress with diazotrophic chemostat cultures adapted to 68 μM O_2. When glucose-limited diazotrophic chemostat cultures of *A. vinelandii*, adapted to a dissolved oxygen concentration of 68 μM, were subjected to a sudden increase in the oxygen concentration, nitrogenase activity was switched off. Nitrogenase activity was switched on as soon as the oxygen stress was released. As demonstrated before, the degree of switchoff depended on the level of the oxygen stress (Table 1) (6, 13). Since nitrogen fixation requires a large amount of energy, one might expect a release of this energy-draining effect as soon as nitrogenase activity is inhibited. The results of ATP measurements, however, revealed that the opposite was true; oxygen stress resulted in a decrease of the cellular ATP pool, which, like switchoff of nitrogenase, became measurable within 1 min of stress (Fig. 1). Obviously, the decrease of the ATP level was slower at the lower stress (Fig. 1A) than at the higher one (Fig. 1B). When the oxygen stress was released, ATP increased to the cellular level before stress. For chemostat cultures, changes (dX/dt) in biological parameters (X) over time (t) are described by the equation $dX/dt = (\mu - D)X$,

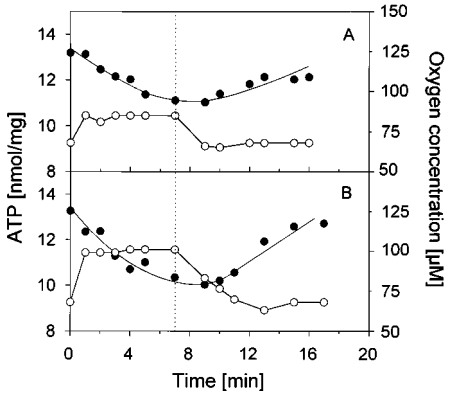

FIG. 1. Effect of oxygen stress on the cellular ATP concentration (●) of diazotrophic chemostat cultures of *A. vinelandii*. After adaptation to 68 μM O_2 (time zero) the oxygen concentration (○) was increased to 85 (A) or 104 (B) μM O_2 for 7 min. Dotted line, time at which the oxygen stress was released. The dilution rate was 0.15 h^{-1} with 0.3% glucose as the growth-limiting substrate.

where D and μ represent the dilution rate and the rate constant at which a given parameter changes, respectively. Under steady-state conditions, μ equals D. If μ differs from D, the level of a given parameter either increases or decreases until a new steady state is reached. Integration of the above equation yields $\mu = D + (\ln X_t - \ln X_0)/(t - t_0)$. Since the term μ is usually employed to denote the growth rate constant, we will use the term μ_{ATP} to refer to the rate at which the cellular ATP level changed. On this basis, the above equation was used to quantify the rate constants at which the cellular ATP levels decreased during oxygen stress. The data in Table 1 show that μ_{ATP} became more negative when oxygen stress was increased. Nitrogenase activity was partially inhibited at the lowest stress level and completely switched off already at the intermediate one. From μ_{ATP} and the steady-state ATP level, the specific rates at which the steady-state ATP level decreased were calculated. The results shown in Table 1 allow comparison of μ_{ATP} as well as the specific rates of ATP disappearance under stress with the corresponding rates required to keep the cellular ATP level constant under steady-state conditions. A dependence of the decrease of the ATP pool on the activity of nitrogenase is suggested by the fact that cultures adapted to 68 μM O_2 and dilution rates of 0.06, 0.09, and 0.15 h^{-1} exhibited nitrogenase activities of 6.5, 9.8, and 16.6 nmol of N_2 fixed per min per mg of protein, respectively. When the oxygen concentration was increased to 85 μM, the respective ATP pools decreased at rates of -9.4, -15.5, and -18.4 nmol/h/mg of protein.

A. vinelandii is known to at least partially switch off nitrogenase activity when exposed to a sudden increase in the concentration of ammonium (14). In contrast to the oxygen stress and in agreement with a previous report (23) a slight increase in the cellular ATP pool could be observed under the conditions of the present investigation.

Oxygen stress with ammonium-assimilating cultures. Bühler et al. (1, 2) showed that ammonium-assimilating chemostat cultures increased the rate of cellular respiration when growing under steady-state conditions at increasing C/N ratios. The respiratory rate reached constancy when active nitrogenase proteins were expressed. With cultures adapted to 68 μM O_2, the threshold C/N ratio for the occurrence of cellular nitrogenase activity was 48 (atoms of C per atom of N). On this basis we asked if the oxygen stress affected the ATP pool only in cultures expressing nitrogenase activity or if the observed effect was independent of the type of N metabolism. To answer this question, cultures adapted to three different C/N ratios at 68 μM O_2 were subjected to an oxygen stress of 104 μM O_2 (Fig. 2). At a C/N ratio of 6, cells growing at the expense of ammonium exhibited an in situ respiratory activity of 0.35 μmol of O_2 consumed per min per mg of protein. Increasing the oxygen concentration to 104 μM had no effect on the cellular ATP pool (Fig. 2A). The C/N ratio of 36 was still below the C/N ratio required for nitrogenase expression (2). A culture grown at this C/N ratio consumed oxygen in situ at a rate of 1.45 μmol/min/mg of protein. Nevertheless, the data of Fig. 2B show that the oxygen stress had no significant effect with respect to the cellular ATP pool. A third chemostat culture assimilating ammonium and, at the same time, fixing dinitrogen was established at a molar C/N ratio of 60. In this culture, the in situ respiratory activity was 1.5 μmol of O_2 per min per mg of protein and dinitrogen was fixed at a rate of 3 nmol/min/mg of protein. Under these conditions, the oxygen stress led to a clear-cut decrease of the cellular ATP level (Fig. 2C). It should also be noted that the ATP pool decreased to a level typical of the two cultures which did not fix dinitrogen or express nitrogenase.

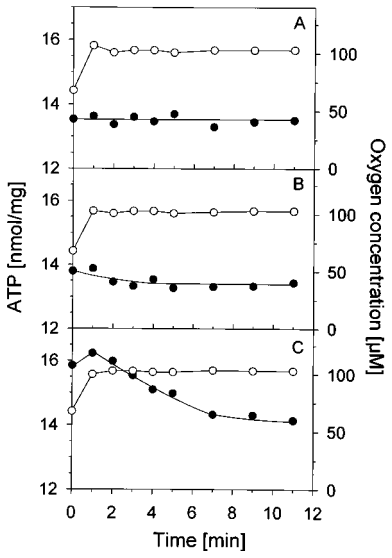

FIG. 2. Effect of oxygen stress on the cellular ATP concentration (●) in chemostat cultures of *A. vinelandii* adapted to 68 μM O_2. The cultures were growing with 2.5 mM ammonium and different glucose concentrations to obtain C/N ratios (atoms of C per atom of N) of 6 (A), 36 (B), and 60 (C). At C/N ratios of 6 and 36, the cultures were ammonium assimilating, while at a C/N ratio of 60 the culture additionally fixed dinitrogen. The oxygen concentration (○) was increased (at time zero) to 104 μM for 11 min.

Oxygen stress with mutant MK5 of *A. vinelandii*. Mutant strain MK5 is impaired in aerotolerant diazotrophic growth (11). It has been proposed that this resulted from the inability of MK5 to form the terminal cytochrome *bd* oxidase. In our hands, the strain was able to grow diazotrophically in glucose-limited oxygen-controlled chemostat culture at up to 18 to 23 μM O_2. On this basis, diazotrophically growing chemostat cultures of strain MK5, the parent strain (UW136) of MK5, and the wild-type strain OP were subjected to oxygen stress experiments after adaptation to 2.3 μM O_2. A sudden increase in the oxygen concentration from 2.3 to 38 μM O_2 completely switched off nitrogenase activity. Interestingly, for the first minute of stress the ATP pool of each strain increased. Subsequently, however, the ATP pool showed essentially the same decrease as that for strain OP adapted to 68 μM O_2. The ATP pools of MK5, UW136, and OP recovered and nitrogenase was switched on upon termination of the oxygen stress.

The switchoff of nitrogenase represents a short-term response to oxygen stress. Adaptation to increased oxygen is assumed to require increased activity of uncoupled respiration and, consequently, enhanced synthesis of the terminal oxidase containing cytochrome *d* (8, 10). Adaptation from 2.3 to 38 μM O_2 was tested with diazotrophic chemostat cultures of strains OP, UW136, and MK5. In the course of 9 h the respiratory activity of UW136 increased by a factor of 2.8 (Fig. 3A). The cell protein content of the culture decreased during the first 7 h (Fig. 3B). After that time a new constant steady-state biomass level was reached, indicating reactivation of nitrogenase. The specific rate of in situ nitrogen fixation before stress and after adaptation to increased oxygen was 16.6 nmol of dinitrogen fixed per min per mg of protein. According to the results shown above, oxygen stress led to a decrease in the ATP pool. The minimum cellular ATP level was reached after 0.25 h of stress. Subsequently, the ATP level started to slowly increase and eventually, after 5 h, reached the original value before stress (Fig. 3B). Essentially the same response was observed with strain OP (not shown). Mutant MK5, however, responded

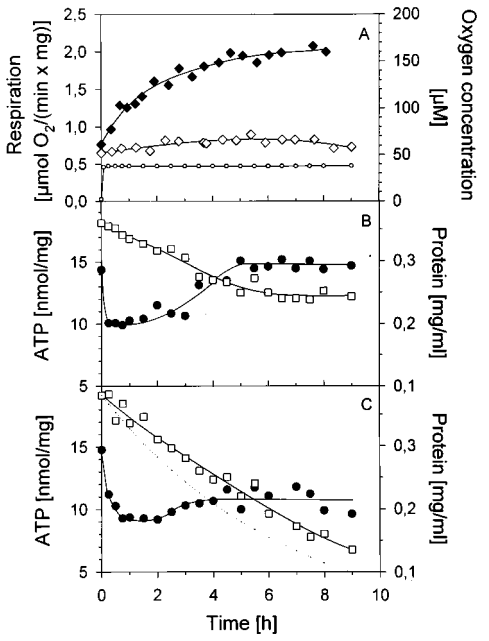

FIG. 3. Effect of a long-term increase (starting at time zero) in the oxygen concentration from 2.3 to 38 μM O_2 in chemostat cultures of strains MK5 and UW136 of *A. vinelandii* growing diazotrophically at a dilution rate of 0.15 h^{-1}. Respiratory activities of MK5 (◇) and UW136 (◆) were measured with samples taken from the cultures (A). ATP concentrations (●) and biomasses (□) of UW136 (B) and MK5 (C) were measured after the oxygen concentration (○) was increased for 9 h as shown in panel A. Dotted line in panel C, theoretical washout at $D = 0.15$ h^{-1}.

quite differently to this long-term increase in ambient oxygen (Fig. 3C). First of all, the mutant was washed out of the chemostat culture, indicating that it was unable to fix sufficient amounts of dinitrogen to support growth. Yet, as depicted in Fig. 3C, the culture was washed out at a rate slower than the dilution rate of 0.15 h^{-1}. Secondly, in agreement with its inability to form cytochrome *d*, MK5 was unable to significantly increase the respiratory activity (Fig. 3A). Following a steep decrease during the first 0.25 h of exposure to 38 μM O_2, the ATP pool attained a new, constantly low size, which increased only slightly, if at all, in the course of 9 h (Fig. 3C). Thus, in contrast to its parent strain, mutant MK5 was not able to readjust the ATP pool to the level before stress. The hypothesis that this lack was the result of decreased consumption of the energy-regenerating substrate can be excluded because measurements of glucose concentrations showed that glucose consumption increased while the cells were washed out of the culture.

The fact that MK5 was not washed out at a rate corresponding to the dilution rate suggested that some residual activity of nitrogenase was still contributing to biomass production. As a matter of fact, 6.5 h after the dissolved-oxygen concentration was increased from 2.3 to 38 μM the in situ activity of nitrogenase in the culture was 3.1 nmol of acetylene reduced per min per mg of protein. This activity was 10% of the steady-state nitrogenase activity attained at 2.3 μM O_2.

DISCUSSION

The nitrogenase reaction requires hydrolysis of at least 16 mol of magnesium-ATP per mol of dinitrogen fixed. On the basis of data obtained under conditions of ammonium-induced switchoff, this suggests that the ATP pool should increase as soon as nitrogenase is switched off (23). However, the data of the present investigation revealed that upon oxygen stress the cellular ATP pool decreased in proportion to the stress. Importantly, cellular ATP levels decreased at rates which were more than 10 times higher than the rates required to keep the ATP pool constant under steady-state conditions. These high rates seem to be very well suited to explain the fast occurrence of the switchoff reaction.

It is known that nitrogenase may reduce not only dinitrogen but also oxygen (22). This leads us to propose the following working hypothesis on the sudden decrease of the cellular ATP pool upon oxygen stress. Since reduction of oxygen by reduced nitrogenase requires energy and reducing equivalents, it may be postulated that, upon oxygen stress, electron transport phosphorylation decreases and ATP utilization by nitrogenase increases. On this basis, the fact that ATP levels decreased only to a limited extent can be explained as follows: utilization of the electron donor as well as of energy should be expected to come to a halt as soon as nitrogenase reaches the redox state required for the formation of the protected but inactive three-component complex. From these interpretations it follows that the switchoff of acetylene reduction by nitrogenase was the result of two processes, namely, preferential reaction with oxygen followed by the formation of the protected complex. Moreover, it follows that the decrease of the ATP pool depended on the presence of an active nitrogenase. This conclusion is supported by the following results: (i) the ATP level did not decrease when ammonium-assimilating cultures, in which nitrogenase was not derepressed, were subjected to a sudden increase in the dissolved-oxygen concentration, and (ii) the ATP pool decreased at a rate proportional to the activity of nitrogenase.

In order to further test the above interpretation, we employed mutant MK5, lacking cytochrome *bd* (11) and, therefore, the assumed uncoupled branch of the respiratory system of *A. vinelandii* (9). Upon reversible oxygen stress, this mutant showed the same response with respect to the cellular ATP level as the two control strains of *A. vinelandii*. Obviously, mutant MK5 was not impaired in conformational protection of nitrogenase. This implies that, in the wild type, the decrease of the ATP level was not the result of switching from the coupled to the uncoupled branch of the respiratory system. Most importantly, however, and in contrast to the two control strains, mutant MK5 was unable to recover from stress after prolonged incubation in the presence of increased oxygen. When subjected to the same stress conditions, strains UW136 and OP were able to increase the ATP pool after a period of about 4 h to the size before stress while mutant MK5 showed only a slight increase in the cellular ATP content. Obviously, this level was sufficiently high to reactivate nitrogenase but not high enough to support diazotrophic growth. The results obtained with the mutant lead to the conclusion that, because of its inability to form cytochrome *bd*, MK5 was unable to adapt its energy metabolism to increased oxygen concentrations. Thus, we are left with the apparent paradox that the branch assumed to be uncoupled is required for an adaptation of the energy metabolism to increased aeration. This paradox may be resolved if it is remembered that two coupling sites (I and II) are assumed to be located before the branching point of the respiratory system of *A. vinelandii* while a third coupling site (III) is contributed by the branch terminated by cytochrome a_1o (9). Moreover, a mutant (DL10) deficient in cytochrome *o* was shown to be capable of the same aerotolerant diazotrophic growth as the wild type, although its respiratory activity was reduced by 55% (15). On this basis it may be assumed that sufficiently large amounts of energy can be regenerated

through sites I and II, provided the flow of electrons through the initial section of the respiratory chain is high enough. It follows that in mutant DL10 the uncoupled branch terminated by cytochrome *bd* is required to increase electron flow and, thus, the rate of ATP synthesis at sites I and II. In mutant MK5, however, lacking the uncoupled branch, the flow of electrons and, consequently, ATP regeneration remain too low to reactivate diazotrophic growth at a high level of oxygen. All of these results suggest that regeneration of ATP rather than consumption of oxygen at the cell's surface is important in protecting nitrogenase activity of *A. vinelandii* against oxygen.

The unexpected occurrence of a drastic decrease of the cellular ATP pool upon oxygen stress may be the result of enhanced utilization of energy and reducing equivalents by nitrogenase reacting with oxygen. Adaptation to high levels of oxygen requires participation of the uncoupled branch of the respiratory system, facilitating an increase in electron flow through the respiratory chain and, thus, ATP regeneration via the two coupling sites of the initial section of the respiratory chain. High rates of supply of both the electron donor and energy are required to keep the enzyme in a reduced state under aerobiosis. This, in turn, is a prerequisite for dinitrogen fixation as well as for autoprotection of nitrogenase (22). The latter process may be assumed to contribute significantly in addition to uncoupled respiration to the high maintenance requirements typical of azotobacters (12).

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