Oxygen Regulation of Nitrate Transport by Diversion of Electron Flow in *Escherichia coli*

(Received for publication, July 19, 1990)

Kimberly S. Denis, Fiona M. Dias, and
John J. Rowe

From the Department of Biology, University of Dayton, Dayton, Ohio 45469-2320

Anaerobic nitrate respiration is regulated by oxygen at the level of nitrate transport; however, the mechanism of O2 inhibition is unknown. Potentially, oxygen could inhibit directly by causing conformational changes in the porter system or indirectly through diversion of electron flow from the nitrate reductase complex to oxygen reduction. Inhibition due to electron diversion implies that nitrate reduction is required for nitrate transport. In this regard, nitrate uptake and its regulation by oxygen were studied in mutants of *Escherichia coli* (strain AN386) deficient in cytochrome d (RG98), cytochrome o (RG101), and a mutant deficient in both cytochrome d and cytochrome o (RG99). Respiratory nitrate uptake in RG99 was highly resistant to the effects of oxygen supporting the indirect mechanism of electron diversion in oxygen regulation. Nitrate transport in RG98 and RG101 is highly sensitive to oxygen; these mutants exhibited 81 and 85% inhibition, respectively, which is similar to inhibition in the wild type. These results indicate that during nitrate respiration, O2 inhibits transport by limiting the supply of electrons to the nitrate reductase complex.

*Escherichia coli* can respire aerobically at the expense of oxygen or anaerobically using nitrate as a terminal electron acceptor via a membrane-bound nitrate reductase enzyme complex. Oxygen reduction by cytochrome o and d occurs on the inner face of the cytoplasmic membrane as oxygen diffuses through the membrane (1). Nitrate reduction also occurs on the inner aspect of the membrane, but the exact mechanism by which nitrate enters the *E. coli* cell is unknown (2). Both forms of respiration produce a proton gradient, which is subsequently utilized directly as a source of energy or transformed ATP by the membrane-associated ATPase (3, 4).

Anaerobic nitrate respiration is regulated at the level of gene transcription (2, 5) or subsequent to gene expression at the level of nitrate transport (6). Anaerobiosis depresses the synthesis of the proteins involved in nitrate respiration, and the expression of these genes is further enhanced by the presence of nitrate (2). After the induction of the nitrate respiratory system, nitrate reduction is immediately and reversibly regulated by oxygen. It was originally demonstrated in denitrifying organisms (7–9) and later in *E. coli* (6, 10) that oxygen acts by preventing the transport of nitrate to the site of its reduction. The mechanism of this inhibition is vague and has not been defined.

One possible model for oxygen inhibition of transport is an oxygen-sensitive nitrate porter system. Since the catalytic site of nitrate reductase is located on the inner face of the membrane, a nitrate transport system could be envisioned where oxygen induces a conformational change in the uptake protein thereby preventing nitrate uptake into the cell. In this model, active sulphydryl groups in the transport protein might be required for nitrate transport. Thus electrons would be diverted from the nitrate respiratory chain to oxygen respiration, a more energy efficient process.

If the transport of nitrate was dependent on the presence of intracellular nitrate as in a nitrate/nitrite antiport system, a second model can be envisioned which is based on the assumption that nitrate transport is dependent on nitrate reduction. Therefore a diversion of electrons from the nitrate reductase complex would prevent nitrate uptake and alter the redox state of the membrane. In the presence of oxygen, electrons preferentially flow to cytochrome o or d which reduce oxygen to water; nitrate reductase is therefore starved for electrons, nitrate reduction ceases, and the intracellular level of nitrite required for the transport of nitrate is therefore limited.

The present investigation focuses on the effect of oxygen on nitrate uptake in mutants of *E. coli* defective in the ability to use oxygen as a terminal electron acceptor for respiration. Therefore, if oxygen regulates anaerobic nitrate respiration through electron diversion to oxygen respiration, this mutant lacking both cytochrome o and d activity should be resistant to changes in oxygen tension during nitrate transport.

**EXPERIMENTAL PROCEDURES**

Organisms and Media—*E. coli* AN386 and three TnlO insertion mutants were obtained for this study from Dr. Robert Gennis, University of Illinois. RG98 is deficient in cytochrome d, RG101 lacks the cytochrome o complex, and a double mutant (RG99) is deficient in both terminal cytochrome oxidases thus preventing aerobic respiration in this organism (11).

All organisms were grown on tryptic soy broth containing 0.25% dextrans and supplemented with 0.1% yeast extract. Potassium nitrate (20 mm) was added to induce nitrate respiratory systems. Tetracycline (15 μg/ml) was incorporated in the medium for growth of the mutants.

Growth Conditions—For all physiological studies, cells were grown in the following manner. A 1-liter Erlenmeyer flask filled to the neck with media was inoculated with a large loopful of the appropriate organism taken from a 48-h culture maintained on tryptic soy slants. The flask was then sealed with a stopper which allowed for gas release, flushed with argon to establish anaerobic conditions, and incubated at 37 °C for 12–16 h. To prevent clumping, the cultures were continuously stirred with a magnetic stir bar.

The cells were harvested by centrifugation (8000 × g) at room temperature for 10–15 min, washed twice with 20 mM Tris-HCl buffer (pH 7.4), and resuspended to a final concentration of 0.75 g, wet weight, per 50 ml of Tris-HCl buffer. The cell suspension was placed in a 50-ml glass reaction vessel with ports for substrate addition and gas flushing. The system was continuously flushed with argon to maintain anaerobic conditions.

The cells were harvested by centrifugation (8000 × g) at room temperature for 10–15 min, washed twice with 20 mM Tris-HCl buffer (pH 7.4), and resuspended to a final concentration of 0.75 g, wet weight, per 50 ml of Tris-HCl buffer. The cell suspension was placed in a 50-ml glass reaction vessel with ports for substrate addition and gas flushing. The system was continuously flushed with argon to maintain anaerobic conditions.

Nitrate Uptake and O2 Regulation Studies—For all physiological studies, 10 mM glucose was added to the reaction vessel as an energy source. The reaction was initiated by adding KNO3 to a final concentration between 400 and 800 μM. Changes in nitrate concentration were monitored using an Orion nitrate electrode (model 93-07) in conjunction with a double junction reference electrode (model 90-02).

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
as previously described (8). To investigate O2 regulation, air was bubbled through the reaction vessel at 120-150 ml min⁻¹ for 10 min after an initial rate of anaerobic uptake had been established (7 min). Anaerobiosis was then re-established by flushing the system with argon. Rates of nitrate uptake were calculated and expressed in nanomoles of NO₃⁻ min⁻¹ mg protein⁻¹.

**Protein Determinations**—The Coomassie Blue method of protein quantitation as described by Spector (12) was utilized to determine protein content in the cell suspensions.

**RESULTS AND DISCUSSION**

The effect of saturating concentrations of oxygen on nitrate uptake is depicted in Figs. 1 and 2. Under aerobic conditions, nitrate uptake is almost completely inhibited in the wild type (AN386) (Fig. 1A). When anaerobic conditions are re-established, uptake continues once all oxygen has been consumed. This inhibition is both immediate and reversible indicating that nitrate-respiring organisms divert electron flow to a more energy-efficient system of oxygen respiration. In RG99, the mutant deficient in both cytochrome o and d, nitrate uptake is scarcely inhibited when oxygen is introduced into the system and continues at a rate which is at least 60% of the anaerobic rate (Fig. 1B). The effect of oxygen on nitrate uptake in the cytochrome o mutant (RG101) and the cytochrome d mutant (RG98) is illustrated in Fig. 2. Both are still quite sensitive to the effects of oxygen. Although cytochrome d is known to exhibit a greater affinity for oxygen since it is predominant under low oxygen tensions (11), both mutants showed inhibition similar to that which is seen in the wild type AN386.

The effect of oxygen on rates of nitrate uptake in E. coli AN386 and the cytochrome mutants is summarized in Table I. Oxygen inhibition is almost complete in the wild type (AN386), cytochrome d mutant (RG98), and the cytochrome o mutant (RG101) which exhibit approximately 80% oxygen inhibition; however, in the double mutant RG99, O2 exerts substantially less inhibition of nitrate uptake. The resistance of nitrate transport to the effects of oxygen in the cytochrome d, o mutant (RG99) is quite interesting. If simple diversion of electrons from nitrate reductase to O₂ was the entire explanation for O₂ inhibition of nitrate transport then we would expect that this mutant should be 100% resistant to the effects of oxygen. The data indicate that the mutant is not completely resistant to the effects of O₂ (Table I), suggesting that more than one mechanism for O₂ inhibition of transport exists or

![Fig. 1. Nitrate uptake in E. coli AN386 and cytochrome d,o mutant RG99. Uptake of nitrate into the cell is monitored via disappearance of extracellular nitrate in the reaction vessel. O₂ inhibition in the wild type (A) is almost complete when air saturates the cell suspension (10 min) and suppresses nitrate uptake. In the double mutant RG99 (B), O₂ causes minimal inhibition nitrate transport. Anaerobic nitrate uptake resumes in both organisms once all oxygen has been consumed.](http://www.jbc.org/)

![Fig. 2. Nitrate uptake in cytochrome o mutant RG98 and cytochrome d mutant RG101. Nitrate uptake and O₂ regulation studies were performed on mutants deficient in a terminal cytochrome oxidase. Both RG98 (A) and RG101 (B) are highly sensitive to the effects of oxygen, exhibiting equal inhibition. This inhibition is reversible, and nitrate uptake resumes when anaerobiosis is reestablished.](http://www.jbc.org/)

![Table I](http://www.jbc.org/)

| Organism     | Anaerobic | Aerobic | O₂ inhibition |
|--------------|-----------|---------|---------------|
| AN386        | 103.2     | 19.4    | 81            |
| RG101        | 120.2     | 25.3    | 79            |
| RG98         | 108.8     | 16.9    | 71            |
| RG99         | 136.9     | 81.0    | 41            |

![Table II](http://www.jbc.org/)

| Energy source | O₂ inhibition |
|---------------|---------------|
| Glucose       | 81            |
| Succinate     | 80            |
| Malate        | 84            |
| Formate       | 57            |
that the inhibition is mediated by the redox state of the membrane, the latter of which has been documented in lactose transport (14). Another interesting note is that the anaerobic rate of nitrate uptake in the cytochrome d and o mutant (RG99) consistently shows a greater rate of uptake than the wild type (AN386).

One potential mechanism for an oxygen-sensitive porter might be through essential sulphydryl groups which may be required for nitrate transport. In order to test this possibility, nitrate uptake was measured with concentrations of sulphydryl inhibitors N-ethylmaleimide and iodoacetate ranging from 10 to 50 μM. However, these inhibitors showed no effect on nitrate uptake or oxygen regulation in the wild type or the double mutant RG99 (data not shown) suggesting that an oxygen-sensitive nitrate porter system may not be a viable model.

Another interesting observation is the effect of electron donors on oxygen inhibition of nitrate transport. John's study of nitrate reduction in membrane vesicles of E. coli (13) indicated that the electron source for nitrate reduction had an effect on oxygen inhibition. We examined this effect in whole cell suspensions to determine if the site at which electrons feed into the electron transport chain affects oxygen regulation of nitrate uptake. Nitrate reduction in whole cells energized by formate is much more resistant to the effects of oxygen than if glucose, succinate, or malate are used (Table II). This effect may be a result of how and where formate can feed electrons into the electron transport chain. It could also reflect various redox states the membrane might assume when different reduced compounds serve as the primary sources of electrons for respiration.

From these preliminary experiments, it was demonstrated that anaerobic nitrate uptake is much more resistant to oxygen in the double mutant RG99 which is unable to utilize oxygen as a terminal electron acceptor for respiration. The effects of oxygen that were observed on nitrate uptake in this organism were immediate and completely reversible. Treatment of cell suspensions with sulphydryl inhibitors did not affect the rate of anaerobic nitrate uptake which indirectly supports the proposed model of oxygen regulation through electron diversion. Furthermore, the degree of oxygen inhibition appears to be regulated by the energy source in the system. This interesting effect is best illustrated when formate is used as an electron donor. Therefore, it appears that oxygen inhibition is probably through the diversion of electrons from nitrate reductase to oxygen thereby limiting the reduction of nitrate which is required for transport.

REFERENCES

1. Gennis, R. B. (1987) FEMS Microbiol. Rev. 46, 387-390
2. Stewart, V. (1988) Microbiol. Rev. 52, 190-232
3. Boonstra, J., and Konings, W. N. (1977) Eur. J. Biochem. 78, 361-366
4. Mitchell, P. (1961). Nature 191, 144-145
5. Showe, M. K., and DeMoss, J. A. (1968) J. Bacteriol. 95, 1305-1313
6. Hernandez, D., and Rowe, J. (1988) J. Biol. Chem. 263, 7937-7939
7. Alefounder, P., and Ferguson, S. (1980) Biochem. J. 192, 231-240
8. Hernandez, D., and Rowe, J. (1987) Appl. Environ. Microbiol. 53, 745-750
9. Kuzera, J., Karlousky, P., and Dadak, V. (1981) FEMS Microbiol. Lett. 12, 391-394
10. Noji, S., and Taniguchi, S. (1987) J. Bacteriol. 161, 123-127
11. Au., D., Lorence, R. M., and Gennis, R. B. (1985) J. Bacteriol. 161, 123-127
12. Spectre, T. (1978) Anal Biochem. 86, 142-146
13. John, P. (1977) J. Gen. Microbiol. 98, 231-238
14. Konings, W., and Robillard, G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5480-5484
Oxygen regulation of nitrate transport by diversion of electron flow in Escherichia coli.

K S Denis, F M Dias and J J Rowe

J. Biol. Chem. 1990, 265:18095-18097.

Access the most updated version of this article at http://www.jbc.org/content/265/30/18095

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/30/18095.full.html#ref-list-1