Inhibition of aerobic respiration and dissimilatory perchlorate reduction using cyanide

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Received 18 June 2004; received in revised form 23 August 2004; accepted 23 August 2004

First published online 11 September 2004

Edited by S. Silver

Abstract

The effect of low concentrations of cyanide on dissimilatory perchlorate and chlorate reduction and aerobic respiration was examined using pure cultures of Azospira sp. KJ. Cyanide at a concentration of 38 μM inhibited cell growth on perchlorate, chlorate and molecular oxygen, but it did not inhibit the activity of chlorite dismutase. When oxygen accumulation was prevented by adding an oxygen scavenger (Oxyrase or L-cysteine), however, cells completely reduced perchlorate in the presence of cyanide. It was concluded that the inhibition of dissimilative perchlorate reduction by cyanide at this concentration was a consequence of oxygen accumulation, not inhibition of the enzymes used for perchlorate reduction. This finding on the effect of cyanide on respiratory enzymes provides a new method to control and study respiratory enzymes used for perchlorate reduction.

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Keywords: Cyanide; Inhibition; Perchlorate reduction; Respiration pathway

1. Introduction

Perchlorate (ClO₄⁻) has been detected in impacted ground waters generally at levels of 0.5–2 μM (50–200 μg L⁻¹) primarily at sites near its production and use as a solid propellant for rockets, missiles, and fireworks [1]. Due to its effect on hormone production by the thyroid, the US EPA proposed a draft reference dose of 0.03 μg kg⁻¹ of body weight per day, which could produce a drinking water equivalent level of 1 μg L⁻¹ [2]. The National Academy of Science is currently reviewing the risk assessment study [3].

Rikken et al. [4,5] demonstrated that perchlorate reduction required an enzyme, chlorite dismutase, that produced oxygen from chlorite (ClO₂⁻) and proposed a three-step mechanism of perchlorate reduction in which chlorate (ClO₃⁻), chlorite, and dissolved oxygen were sequentially produced. This pathway, which is now widely accepted for bacterial respiration using perchlorate and chlorate, is: ClO₄⁻ → ClO₃⁻ → ClO₂⁻ → O₂ + Cl⁻ [5–10]. The first two steps are catalyzed by perchlorate reductase, and are relatively slower than the last step [5,11]. Oxygen plays a critical role in perchlorate reduction. Oxygen is evolved during chlorite disproportionation but the same bacteria rapidly scavenge oxygen to low concentrations [5]. High concentrations of oxygen immediately inhibit perchlorate reduction [11], and perchlorate reductase has a half life of 2–3 days in the presence of oxygen [11]. Therefore, perchlorate-respiring bacteria must maintain an aerobic respiration pathway during perchlorate reduction in order to maintain a low concentration of dissolved oxygen [12].
2. Materials and methods

2.1. Bacterium and media

Azospira sp. KJ (formerly Dechlorosoma sp. KJ; ATCC BAA-592), originally isolated from a perchlorate-degrading packed bed bioreactor [13], was used in all experiments. The basal medium was prepared as previously described [14]. Sodium acetate ($\text{C}_2\text{H}_3\text{O}_2^-$, 34 mM) was added as the electron donor in all experiments. Sodium perchlorate ($\text{ClO}_4^-$, 5 mM) or chlorate ($\text{ClO}_3^-$, 6 mM) was added as the terminal electron acceptor in anaerobic experiments. The pH of the medium was adjusted to 7.0 using NaOH. The anaerobic medium was sterilized by autoclaving, and degassed in an anaerobic glove box (Coy Scientific Products, Grass Lake, MI). The aerobic medium was prepared without the addition of chlorate or perchlorate, and saturated with dissolved oxygen using air.

Cells were harvested during late-log-phase growth (optical density [OD] at 600 nm of 0.3–0.4, equal to $1.74–2.32 \pm 0.04 \times 10^9$ cells/ml), washed once by centrifuging at 5000g at 4 °C for 10 min and suspended in fresh medium to the same OD.

2.2. Culture growth in the presence of $\text{CN}^-$

The concentration of $\text{CN}^-$ needed to inhibit aerobic cell respiration was determined by growing cells on perchlorate and acetate in the presence of $\text{CN}^-$. Washed cell suspensions (0.50 ml each, triplicate samples) were transferred into 23-ml test tubes (final volume 10 ml) containing basal medium, acetate, and perchlorate or chlorate. $\text{CN}^-$ was added at the final concentration of 38 $\mu$M except in one experiment where several concentrations were tested (0.038–31 mM of $\text{CN}^-$). All the experiments were conducted at 25 °C unless stated otherwise. The inhibition of $\text{CN}^-$ on cell growth was monitored intermittently by cell density ($\text{OD}_{600}$), at the end of the experiment by dry weight, and by loss of the electron acceptor (perchlorate or chlorate) and donor (acetate). Concentrations of chlorite and chloride were monitored during cell growth on chlorate. Dissolved oxygen (DO) concentrations were measured at the beginning and the end of experiments using a dissolved oxygen probe (YSI Model 5331, YSI Incorporated, Yellow Springs, OH) and monitor (YSI Model 5300). The probe was calibrated with air-saturated water before each experiment.

In aerobic growth experiments, $\text{CN}^-$ was added at a final concentration of 38 $\mu$M (treatment) or omitted (control). The test tubes were aerated during experiment by shaking tubes on their sides at 150 rpm (Model 3250; Lab-line Instrument Inc., Melrose Park, IL). The inhibition of $\text{CN}^-$ on cell growth was monitored based on cell density ($\text{OD}_{600}$) and loss of the electron donor (acetate).

2.3. Accumulation of DO during anaerobic cell respiration

DO was monitored during anaerobic cell respiration of perchlorate to explore $\text{CN}^-$ inhibition of oxygen utilization. Cell suspensions (5 ml) were inoculated in 130 ml serum bottles (95 ml of medium prepared under anaerobic conditions) with perchlorate and acetate. Cyanide was added during the early log growth phase (38 $\mu$M), and samples were analyzed for OD$_{600}$, DO, perchlorate, and acetate.

2.4. Perchlorate culture growth with $\text{CN}^-$ and chemical oxygen scavengers

Abiotic control experiments were conducted to demonstrate that cyanide did not alter the effectiveness of the two reducing agents to scavenge dissolved oxygen. Oxirase and L-cysteine [14,15], were added from sterile anoxic aqueous stock solutions to the medium saturated with dissolved oxygen at a concentration either used by others (L-cysteine · HCl, 3.2 mM) [15] or at a concentration recommended by the manufacturer (Oxirase, 0.02 ml/ml). Oxygen removal rates were examined in the absence of $\text{CN}^-$ and at $\text{CN}^-$ concentrations of 38 $\mu$M to
1.9 mM. Each oxygen scavenger was added to a 20-ml chamber containing 3 ml of an air-saturated abiotic growth medium, and constantly stirred. CN⁻ was then added 2.5 min after each experiment started. Oxygen removal rates were calculated from the slope of the dissolved oxygen concentration change over time. Free cyanide concentration in medium containing L-cysteine was measured by precipitating cyanide with 59 μM AgNO₃ [16].

Oxyrase and L-cysteine were used to remove dissolved oxygen produced during anaerobic cell growth in the presence of CN⁻/C₀. In experiments using L-cysteine, cells were pre-acclimated to this chemical in order to avoid lag times [14]. The test tubes containing one of the reducing agents and no CN⁻ served as controls on the effect of the reducing agents on cell growth. The inhibition of CN⁻ on cell growth in the presence of oxygen scavenger was determined by monitoring cell density (OD₆₀₀) and loss of the electron donor (acetate).

2.5. Chlorite dismutation in the presence of CN⁻

Cyanide inhibition of chlorite dismutase activity was determined by measuring the rate of oxygen evolution during chlorite disproportion in the presence CN⁻ using anaerobically grown cultures. Cell suspensions (0.3 ml) were added to a 20-ml YSI DO chamber containing 2.7 ml of phosphate buffer and 38 μM CN⁻, and constantly stirred. Chlorite (30 μl; 10 mM, final concentration) was added 1.5 min after the experiment started and DO measurements taken for 7 min.

2.6. Analytical techniques

Concentrations of perchlorate, chlorate, and acetate were determined with an ion chromatograph (DX500; Dionex, Sunnyvale, CA) equipped with an AS11 column and guard column, a self-regenerating suppressor, and an autosampler. Perchlorate ion was measured using a 100 mM NaOH eluent. For chlorate, chlorite, acetate and chloride measurements, a 10 mM NaOH eluent was used. The minimum detection limit for perchlorate was 0.04 μM. Cell dry weight (DW) was determined using membrane filters (25 mm, 0.2 μm pore diameter; Osmonics Corp., Minnetonka, MI) and a microbalance (Mettler Toledo UMT2, Greifensee, Switzerland).

3. Results

3.1. CN⁻ inhibition of cell growth on ClO₄⁻

Growth of *Azospira* sp. KJ on perchlorate was inhibited by cyanide concentrations of ≥38 μM. In the absence of cyanide, perchlorate was completely reduced with cell growth increasing from OD₆₀₀ = 0.02 (1.2 ± 0.4 × 10⁸ cells ml⁻¹) to a final value of OD₆₀₀ = 0.37 (2.15 ± 0.04 × 10⁹ cells ml⁻¹). With 38 μM cyanide, the final optical density was OD₆₀₀ = 0.06 (3.5 ± 0.4 × 10⁸ cells ml⁻¹) and only 2.5% of perchlorate was degraded. Less than 1% of perchlorate was degraded at cyanide concentrations of 38–7.7 mM, and no perchlorate was removed at a cyanide concentration of 19 mM or more. Cell dry weight of the control lacking cyanide (0.222 mg cell ml⁻¹ culture) was five times the initial cell dry weight (0.041 mg cell ml⁻¹ culture) after incubation for 70 h. Final cell dry weights at cyanide concentrations of 38 μM–3.8 mM were the same as that of the control. Cell dry weights were lower (0.025–0.017 mg cell ml⁻¹, respectively) when 19 or 31 mM of cyanide were used.

The final DO concentrations of all samples containing cyanide were similar to that of the initial sample (25–26 l M), indicating a lack of aerobic cell respiration in the presence of 38 l M or more of cyanide. However, the final DO concentration of the control was <3 μM (initial DO 25 μM).

3.2. CN⁻ inhibition of cell growth on ClO₃⁻ or O₂

Cyanide at 38 μM inhibited cell growth on chlorate or molecular oxygen (Fig. 1). Chlorate was completely degraded by the control. However, only 3.2 ± 0.3% (0.27 ± 0.03 mM) of chlorate was degraded in the culture containing cyanide, while 0.25 ± 0.05 mM of acetate was consumed and 0.31 ± 0.06 mM of chloride was not detected.

![Fig. 1. Inhibition of CN⁻ on *Azospira* sp. KJ growth with (a) ClO₄⁻ or (b) DO. Solid symbols, controls lacking CN⁻; open symbols: cultures containing CN⁻ (38 μM).](https://academic.oup.com/femsle/article-abstract/239/2/229/622083)
was generated. The stoichiometric ratio of chlorate to chloride was 0.87:1, similar to that of the control (0.89:1). No chlorite was detected in any samples indicating that chlorite dismutase activity was unaffected by 38 \( \mu \text{M} \) cyanide. The final DO concentration of the chlorate control was much lower than that of initial sample, as observed for perchlorate. The DO level of the sample with cyanide was the same as that of initial sample. For the aerobic culture, no DO was used by bacteria in the presence of cyanide.

3.3. DO accumulation in anaerobic cultures treated with \( \text{CN}^- \)

Cyanide inhibited oxygen utilization by anaerobically grown cells. Evolved oxygen accumulated to higher concentrations in anaerobically grown cultures treated with cyanide than in controls lacking cyanide. The concentration of oxygen generated during perchlorate reduction reached 1.6 \( \mu \text{M} \) immediately after the addition of 38 \( \mu \text{M} \) of cyanide during early log growth phase, resulting in the loss of cell growth. However, the DO concentration of the control lacking cyanide was below the detection limit of the DO probe (1.3 \( \mu \text{M} \)). It is known that high concentrations of DO inhibits perchlorate respiration \cite{11}, but these results demonstrate that a very low DO concentration (1.6 \( \mu \text{M} \)) inhibits cell growth.

3.4. Culture growth with \( \text{CN}^- \) and oxygen scavengers

To demonstrate that a lack of cell growth on perchlorate was due to DO accumulation produced by cyanide, reducing agents were added to scavenge oxygen. \textit{Azospira} sp. KJ grew on perchlorate in the presence of a low concentration of cyanide when Oxyrase or L-L-cysteine was added to scavenge oxygen (Fig. 2). In the presence of Oxyrase, cultures containing 38 \( \mu \text{M} \) of cyanide grew at a rate similar to that of the control (no cyanide). Higher concentrations of cyanide inhibited both aerobic respiration and perchlorate reduction. With Oxyrase and 190 \( \mu \text{M} \) of cyanide, cell growth was much slower than that of the control (Fig. 2(a)). At cyanide concentrations of 0.38 mM or more there was no cell growth on perchlorate after 80 h even in the presence of Oxyrase. Cell yields (0.279 mgcellml\(^{-1}\)) using Oxyrase, but no cyanide, were larger than the control (0.216 mgcellml\(^{-1}\)) (Fig. 2(a)) suggesting that ingredients in Oxyrase were used as a substrate for cell growth. Although growth was slower in the presence of a chemical oxygen scavenger, perchlorate was still completely degraded by samples with 190 \( \mu \text{M} \) of cyanide. There was no perchlorate removal at cyanide concentrations >0.38 mM.

When L-cysteine was used to scavenge oxygen, growth was slower in the presence of 38 \( \mu \text{M} \) of cyanide than that of the control (Fig. 2(b)). Perchlorate was completely degraded by cells in the absence of cyanide, or in the presence of 38 \( \mu \text{M} \) of cyanide. With cyanide at 190 \( \mu \text{M} \) or more there was no culture growth and no perchlorate degradation for over 90 h.

The addition of cyanide slowed, but did not inhibit, the ability of the reducing agents to scavenge oxygen. The rate of oxygen removal by Oxyrase was reduced by 45% for 38 \( \mu \text{M} \) of cyanide, and by 91% with 190 \( \mu \text{M} \) of cyanide in media lacking cells (Fig. 3). Cyanide at 190 \( \mu \text{M} \) completely inhibited oxygen removal by Oxyrase (data not shown). Cyanide concentrations of 38 and 190 \( \mu \text{M} \) decreased oxygen removal rates in the presence of L-cysteine by 41% and 80% (Fig. 3), respectively, while cyanide at 0.38–1.9 mM decreased oxygen removal rates by 93% (data not shown). \text{AgNO}_3 precipitation results indicated that 95% of the cyanide was present as a free anion in an abiotic solution containing L-cysteine. Because the activity of the reducing agents is inhibited at higher concentrations of cyanide, it was not possible to examine inhibition of perchlorate respiration separately from aerobic pathways at higher cyanide concentrations via cell growth or oxygen utilization.
3.5. Chlorite dismutation with $\text{CN}^-$

Cyanide at concentrations of $\leq 38 \text{\mu M}$ did not inhibit chlorite dismutation (Fig. 4). Oxygen generation in chlorite-spiked samples (10 mM) was similar to that of the control lacking cyanide. Cyanide concentrations $>0.38 \text{ mM}$, however, partially inhibited chlorite dismutation and concentrations of $>3.8 \text{ mM}$ completely inhibited chlorite dismutation (Fig. 4).

4. Discussion

Cyanide at a concentration of 38 $\mu$M inhibited *Azospira* sp. KJ growth on perchlorate or chlorate (Fig. 1(a)) due to the accumulation of dissolved oxygen, not the loss of perchlorate reductase or chlorite dismutase activities. Aerobic cell growth was completely inhibited with 38 $\mu$M of cyanide (Fig. 1(b)). However, chemical scavenging of DO produced during anaerobic growth allowed anaerobic cell growth in the presence of 38 $\mu$M of cyanide (Fig. 2). Cyanide affects heme-based enzymes, such as chlorite dismutase and cytochrome oxidase, both of which are needed for perchlorate reduction [5]. Cyanide concentrations of $\geq 3.8 \text{ mM}$ were needed to inhibit chlorite disproportionation by strain KJ. This is consistent with a previous report that 20 mM of cyanide inhibited the activity of chlorite dismutase isolated from the dissimilatory perchlorate reducing strain GR-1 [5]. Thus, the lack of anaerobic cell growth on perchlorate in the presence of cyanide at a concentration of 38 $\mu$M was not due to inhibition of chlorite dismutase activity.

The above results provide direct evidence of a branched respiration pathway for perchlorate reduction. While aerobic respiration is inhibited by 38 $\mu$M of cyanide, it is still possible to achieve perchlorate respiration. Thus, there must be a branch point to perchlorate reductase in the electron transport chain used by these bacteria under anaerobic conditions. These findings of the effect of cyanide on the different enzymes used for perchlorate and aerobic degradation will allow further examination of the respiratory pathways used by perchlorate respiring bacteria.

Acknowledgements

We thank Robert Arnold for valuable discussions on branched respiratory pathways and inhibitors, and comments by Simon Silver. This research was supported by a National Science Foundation Grant (BES-0001900).

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