Abiotic Reduction of 4-Chloronitrobenzene to 4-Chloroaniline in a Dissimilatory Iron-Reducing Enrichment Culture

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4-chloronitrobenzene (4-Cl-NB) was rapidly reduced to 4-chloroaniline with half-lives of minutes in a dissimilatory Fe(III)-reducing enrichment culture. The initial pseudo-first-order rate constants at 25°C ranged from 0.11 to 0.19 per minute. The linear Arrhenius correlation in a temperature range of 6 to 85°C and the unchanged reactivity after pasteurization indicated that the nitroreduction occurred abiotically. A fine-grained black solid which was identified as poorly crystalline magnetite (Fe_3O_4) by X-ray diffraction accumulated in the enrichments. Magnetite produced by the Fe(III)-reducing bacterium *Geobacter metallireducens* GS-15 and synthetic magnetite also reduced 4-Cl-NB. These results suggest that the reduction of 4-Cl-NB by the enrichment material was a surface-mediated reaction by dissimilatory formed Fe(II) associated with magnetite.

In anoxic environments, reduction of the nitro group is one of the first steps during the transformation or mineralization of nitroaromatic compounds (11). Since nitroreduction can lead to products of similar or even greater environmental concern, information on the mechanisms and rates of this process is of great interest. A question commonly addressed is whether such reactions occur abiotically, whether microorganisms are involved, or whether both processes are important. It has been shown that a variety of bacteria can reduce nitrosubstituted aromatic compounds (3, 4, 12, 14, 15). However, the biological reductions are considered too slow to account for the rapid transformation rates sometimes observed in natural environments. For example, rapid reduction of the nitro group of the pesticides parathion and methyl parathion with half-lives of only seconds to minutes has been found in anoxic soils and sediments (1, 22, 23). The main abiotic bulk electron donors in anoxic environments are reduced sulfur and iron species (19). Sulfide and sulfide minerals have been found to react with nitroaromatic compounds (17, 24), although at slow rates. The presence of various electron transfer mediators such as quinones and iron porphyrins dramatically increased the reducing activity in homogeneous aquatic systems (5, 6, 17, 21). Studies on the transformation of parathion in soil slurries suggested an abiotic surface-mediated process (23). This indicates that the rapid nitroreduction observed in environmental samples could involve abiotic processes that are catalyzed by electron mediators or that occur at surfaces.

Little is known about the role of inorganic forms of iron(II) with respect to the transformation of xenobiotic compounds. Results of experiments in laboratory columns containing reduced aquifer materials suggest that Fe(II) may be the reductant (16). In this paper, we present evidence that dissimilatory formed Fe(II) associated with magnetite (Fe_3O_4) is responsible for the rapid surface-mediated nitroreduction of

4-chloronitrobenzene (4-Cl-NB) observed in an Fe(III)-reducing enrichment culture.

Anoxic freshwater sediment from Lake Lucerne collected at a depth of 60 m was used as an inoculum for the iron(III)-reducing enrichment. The selective FeOOH medium described by Lovley and Phillips (9) was employed. Acetate (20 mM) served as the carbon and energy source, and Fe(III) was provided as amorphous Fe(III) oxyhydroxide at approximately 90 or 150 mmol of Fe(III) per liter of medium, unless otherwise stated. Serum bottles containing FeOOH medium and sealed with butyl rubber stoppers were amended with 10% inoculum (vol/vol) by syringe. Incubation occurred at 30°C on a rotary shaker (100 rpm).

The nitroreduction assay was carried out under anaerobic conditions at 25°C (if not otherwise stated) in 60-ml serum bottles which were sealed with butyl rubber stoppers. The 14-ml reaction mixture contained MOPS (morpholinepropanesulfonic acid) buffer (70 mM, pH 7.0), 50 µM 4-Cl-NB, and where indicated, an aliquot of an Fe(III)-reducing enrichment culture. During an experiment, the solution was continuously mixed with a magnetic stirrer. A reaction was initiated by adding an appropriate volume of a 0.1 M methanolic solution of 4-Cl-NB to the reaction mixture. The nitroreduction was monitored by periodical sampling by syringe. 4-Cl-NB and possible intermediates and products were analyzed by high-performance liquid chromatography as described previously (6), except that perchloric acid was added to the sample (final concentration, 0.3 M) to stop the reduction. The intermediate 4-chlorohydroxylamine was quantified by the method described previously (5). The reaction could only in the initial phase be described by pseudo-first-order kinetics. The initial pseudo-first-order transformation rate constant k_{obs} was determined by a linear regression of the first datum points of the curve in a plot of ln ($[4\text{-Cl-NB}]_t/[4\text{-Cl-NB}]_0$) versus time, where $[4\text{-Cl-NB}]_t$ and $[4\text{-Cl-NB}]_0$ are the 4-Cl-NB concentrations at time t and time zero, respectively. Acetate concentration was determined by ion chromatography (Metrohm model 690 [Herisau, Switzerland]) with a Hamilton PRP-X300 column. The flow rate was 1.5 ml/min, and the mobile phase was 0.5 mM sulfuric acid. Fe(II), extractable with 0.5 M HCl (20 min at 20°C),

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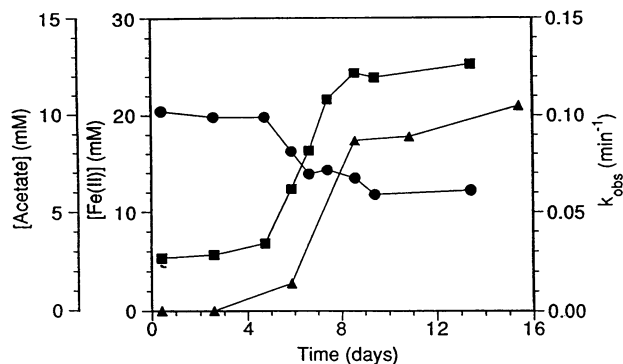
FIG. 1. Acetate consumption, HCl-extractable Fe(II) formation, and nitroreducing activity in an enrichment inoculated with Lake Lucerne sediment. Each reduction assay contained 20% enrichment material (vol/vol). ●, acetate; ■, Fe(II); ▲, k_{obs}.

FIG. 3. Arrhenius plot for the reduction of 4-Cl-NB for a temperature range of 6 to 85°C. The reduction assay mixture contained 5.6 mM Fe(II).

was determined with a phenanthroline colorimetric assay at 510 nm (2).

In an enrichment inoculated with Lake Lucerne sediment, acetate consumption was paralleled by the formation of HCl-extractable Fe(II), which indicated that acetate oxidation was coupled to dissimilatory Fe(III) reduction (Fig. 1). A ratio of only 1:5 between acetate and Fe(II) was found instead of 1:8 according to the equation $CH_3COO^- + 8$ $Fe(III) + 4 H_2O \rightarrow 2 HCO_3^- + 8 Fe(II) + 9 H^+$. This is possibly due to acetate consumption by other processes (e.g., cell growth) and/or the presence of nonextractable Fe(II). Samples of the enrichment culture were taken during the incubation and assayed for nitroreducing activity with 4-Cl-NB. Only samples with accumulated Fe(II) rapidly reduced 4-Cl-NB with half-lives of minutes for the given conditions (Fig. 1). The observed increase in k_{obs} with time can be explained by the increasing amounts of Fe(II) or bacteria present in the cultures. Samples from enrichments incubated with either only acetate or only Fe(III) oxyhydroxide did not show any reducing activity in the time frame investigated. The nitroreduction of 4-Cl-NB proceeded via the 4-chlorohydroxylamine intermediate into the corresponding aniline (Fig. 2). The observed reduction products are in accordance with the scheme of reduction of an aromatic nitrosubstituent commonly assumed to occur in neutral aqueous systems (5). The mass balances indicated
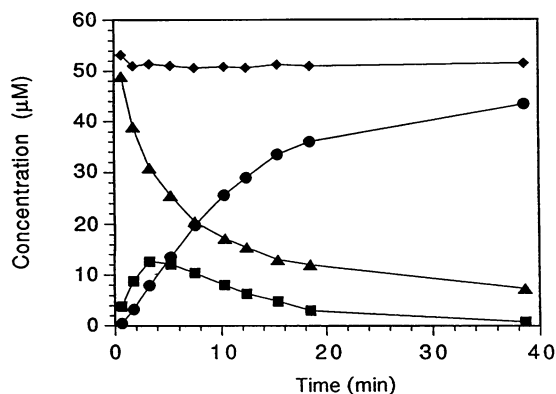

FIG. 2. Reduction pattern of 4-Cl-NB (▲) to 4-chloroaniline (●) via 4-chlorohydroxylamine (■), with the mass balance (◆) indicated. The reduction assay mixture contained 10.6 mM Fe(II).
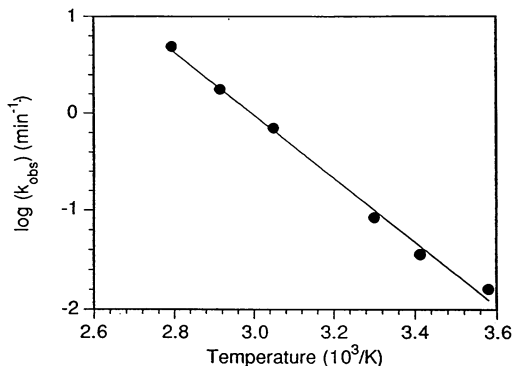
that 4-chloroaniline was the only end product of the reaction (Fig. 2). A linear correlation was observed between the amount of enrichment material in the reduction assay and the initial pseudo-first-order rate constant (data not shown). However, among different enrichment cultures, the initial pseudo-first-order rate constants corrected for the amount of enrichment material present varied by less than a factor of 4. Samples of an enrichment taken shortly after completion of Fe(III) reduction or several months later had the same nitroreducing activities.

Possible components of the enrichment material responsible for the nitroreduction are dissimilatory Fe(III)-reducing bacteria or different Fe(II) species. A pasteurization at 70°C for 30 min did not result in a decrease in reducing activity, which was the first indication of an abiotic process. A complete loss of reactivity was observed after autoclaving at 120°C for 20 min. This demonstrates that this rigorous sterilization procedure often used to show the involvement of biological reactions may also change chemical and physical characteristics of the sediment material (11, 23). Therefore, a chemical reaction cannot be ruled out with this experiment. As depicted in an Arrhenius plot (Fig. 3), the initial pseudo-first-order transformation rate constant for 4-Cl-NB increased exponentially in a temperature range of 6 to 85°C, which also supports an abiotic mechanism. An enzyme-catalyzed reaction would have an activity maximum and would not show activity at temperatures as high as 85°C. The addition of metal complexing agents such as EDTA (0.3 mM) or phosphate (10 mM) to the nitroreduction assay mixture completely inhibited the reduction of 4-Cl-NB. This is additional evidence for an abiotic, Fe(II)-catalyzed process.

The dissimilatory formed Fe(II) accumulated as a fine-grained black solid, and the accumulation stopped when one-third of the Fe(III) was reduced. The solid fraction of a twice-transferred culture was characterized by X-ray diffraction with a Philips 1170 powder diffractometer using CuK_α radiation and a postmonochromator. Poorly crystalline magnetite ($Fe^{II}Fe^{III}Fe^{III}O_4$) was the only detectable product. Removal of particles by filtration with a 0.2 μm-pore-size filter resulted in a filtrate with very little activity. Also, no 4-Cl-NB reduction was observed in a homogeneous synthetic Fe(II) solution [Fe(II)methylsulfonate, 20 mM]. All these findings indicated that dissimilatory formed Fe(II) associated with magnetite was the reductant of 4-Cl-NB in a heterogeneous reaction.

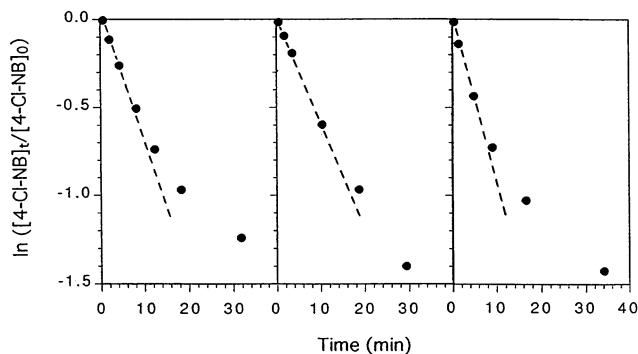*Geobacter metallireducens* GS-15, an anaerobic bacte-

FIG. 4. Plot of ln ($[4\text{-Cl-NB}]_t/[4\text{-Cl-NB}]_0$) versus time for the reduction of 4-Cl-NB by enrichment material (left), material of a *G. metallireducens* GS-15 culture (center), and synthetic magnetite (right). The reduction assay mixtures contained 5.9 mM, 3.2 mM, and 1.7 mM Fe(II), respectively.

rium capable of coupling the complete oxidation of acetate to the reduction of Fe(III) (8), forms magnetite upon reduction of amorphous Fe(III) oxyhydroxide in the FeOOH medium used in this study (10). Samples of such cultures reduced 4-Cl-NB at rates similar to those of samples from the enrichment cultures (Fig. 4). Synthetic magnetite formed by Fe(II) oxidation at neutral pH (20) also reduced 4-Cl-NB (Fig. 4). Autoclaving synthetic magnetite or adding EDTA or phosphate to the reaction mixture completely destroyed the nitroreducing activity. This is in agreement with the results obtained with enrichment material and confirms the possibility that, besides living organisms, other components may be inactivated by this heat treatment.

A direct comparison of the rate constants of the different systems is presently not possible, since the system parameters (e.g., surface area and concentration of reactive sites, etc.) determining the overall reaction rate are not yet known. As can be seen from Fig. 4, in all three systems investigated the reduction of 4-Cl-NB can only in the initial phase be described by first-order kinetics. With increasing time, the reaction rate constant decreased continuously. Although there was still enough Fe(II) present in the reaction mixture, the formation of reactive Fe(II) sites may have become rate limiting. Similar kinetics were found for the reductive dissolution of manganese(III/IV) oxides by substituted phenols and anilines (7, 18). A decrease in transformation rates with time was also observed for the nitroreduction of parathion in samples from soils previously reduced by flooding with water for 60 days (22). More than 50% of the parathion added was reduced within the first 5 s of mixing, whereas only 33% was transformed during the following 30 min. Regeneration of reactive Fe(II) sites in Fe(III)-reducing systems could occur by oxidative dissolution of magnetite or by microbial production of new magnetite.

The results presented above provide strong evidence that Fe(II) species formed during microbial dissimilatory Fe(III) reduction are responsible for the rapid nitroreduction found in the experimental system investigated. The transformation is proposed to be a surface-mediated reduction with a limited number of reactive Fe(II) surface sites associated with magnetite. To our knowledge, this is the first time that it has been indicated that mineral-associated Fe(II) can directly reduce organic pollutants. The results of this study may help to explain experimental data reported in the literature. Ponnamperuma (13) suggested that most of the Fe(II) in rice

paddies is present as hydromagnetite ($Fe_3O_4 \cdot H_2O$) which crystallizes as magnetite if the soil remains anoxic. In such preflooded soils, extremely rapid transformations of nitro-compounds have been found (1, 22). The observed nitroreducing activity was lost in both studies after autoclaving of the anerobic soils, similarly to the results presented above. In sediment systems where nitroreduction of methyl parathion was also inhibited by heat sterilization, chemical sterilization by *m*-cresol only slightly affected the activity, indicating an abiotic process by heat-labile components (23). The importance and occurrence of nitroreductions in the environment by magnetite and other Fe(II)-containing solids is at present under investigation in our laboratory.

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