Reduction of Azo Dyes and Nitroaromatic Compounds by Bacterial Enzymes from the Human Intestinal Tract

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Several anaerobic bacteria from the human intestinal tract are capable of reducing azo dyes and nitropolycyclic aromatic hydrocarbons to the corresponding aromatic amines with enzymes that have azoreductase and nitroreductase activities. The majority of bacteria with these activities belong to the genera *Clostridium* and *Eubacterium.* The azoreductases and nitroreductases from three *Clostridium* strains and one *Eubacterium* strain were studied. Both enzymes were produced constitutively in each of the bacteria; the enzymes from various bacteria had different electrophoretic mobilities. The azoreductases from all of the bacteria had immunological homology, as was evident from the cross-reactivity of an antibody raised against the azoreductase of *C. perfringens* with azoreductases from other bacteria. Comparison of azoreductases and nitroreductases showed that they both had identical electrophoretic mobilities on polyacrylamide gels and reacted with the antibody against the azoreductase from *C. perfringens.* Furthermore, the nitroaromatic compounds competitively inhibited the azoreductase activity. The data indicate that the reduction of both nitroaromatic compounds and azo dyes may be carried out by the same enzyme, which is possibly a flavin adenine dinucleotide dehydrogenase that is synthesized throughout the cell and not associated with any organized subcellular structure. — Environ Health Perspect 103(Suppl 5):17–19 (1995)

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Human Intestinal Bacteria

Azo dyes and nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are two groups of chemicals that are abundant in our environment. Azo dyes are used in the textile, pharmaceutical, food, and cosmetics industries. Nitro-PAHs are ubiquitous environmental contaminants that have been detected in carbon black, photocopier toners, urban air particulates, diesel fuel emissions, used motor oil, barbecued foods, and tea leaves (*1–4*). In mammalian systems, azo dyes and nitro-PAHs are reduced to aromatic amines by enzymes from intestinal bacteria and from the liver. The reduction occurs through cleavage of the azo bridge in azo dyes and conversion of the nitro group to an amino group in the nitro-PAHs (*1,3,5–7*). These reduction processes are accompanied by the decolorization of the azo dyes and nitro-PAHs (*7,8*).

The species of bacteria capable of reducing azo dyes and nitro-PAHs from the human intestinal microflora or other sources can be identified by plating serial dilutions of human feces on brain–heart infusion agar containing either 1-nitropyrene or an azo dye such as Direct Blue 15. Bacterial colonies with azoreductase and nitroreductase activities reduce the dye or 1-nitropyrene on the plate and are recognized by the appearance of clear zones around the colonies. Although several azoreductase- and nitroreductase-producing bacteria are present in the human intestinal tract, the majority belong to the genera *Clostridium* and *Eubacterium* (*7,8*). Predominant anaerobic bacteria with azoreductase and nitroreductase activity found in the human intestinal tract include *Clostridium leptum, Eubacterium* sp., *C. clostridiiforme, C. paraputrificum, Clostridium* sp., and *C. perfringens.*

The conversion of azo dyes and nitro-PAHs to aromatic amines by isolated bacteria can be verified by high pressure liquid chromatography (HPLC) and mass spectrometry. The activity of azoreductase produced by each bacterial isolate can be quantified by following the rate of decrease in the absorbance of azo dye, e.g., Direct Blue 15, at 615 nm with time (*2*). The activity of nitroreductase produced can be determined by measuring the conversion of 4-nitrobenzoic acid to 4-aminobenzoic acid. The 4-aminobenzoic acid is further converted to a diazonium salt by the addition of sodium nitrite under acidic conditions. *N*-(1-Naphthyl)ethylenediaminedihydrochloride (NEDD) converts the diazonium salt to a purple dye, whose color can be measured spectrophotometrically at 540 nm (*9*). Using these techniques, it has been shown that both azoreductase and nitroreductase are produced constitutively in various amounts by several bacteria. Among bacteria isolated from the human intestinal microflora, members of the genus *Clostridium* produce the highest amounts of azoreductase and nitroreductase (*7,8*).

Different forms of azoreductases and nitroreductases are produced by several anaerobic bacteria and can be evaluated by a nondenaturing-gel assay that detects azoreductase and nitroreductase activities on the gel. Ammonium sulfate-precipitated protein, from the spent culture and cell extract of an overnight culture of anaerobic bacteria, is loaded on a nondenaturing polyacrylamide gel under anaerobic conditions. To detect azoreductases after electrophoresis, the gel is incubated with an azo dye (either Nitro Red or Direct Blue 15), flavin adenine dinucleotide (FAD), and NADH. A decolorized band appears at the location of migration of azoreductase protein (*7*).

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For the detection of nitroreductase on the gel, 4-nitrobenzoic acid is substituted for Direct Blue 15 (*10*). The 4-aminobenzoic acid produced by nitroreductase at the location of the nitroreductase band is detected by conversion to a purple dye in the presence of sodium nitrite and NEDD, as described above. Several anaerobic bacteria tested had only one azoreductase and nitroreductase on the gel activity assay and both nitroreductases and azoreductases from various anaerobic bacteria had different electrophoretic mobilities (*8,10*). The antibody against *C. perfringens* was employed to determine the structural and immunological relatedness among azoreductases from *C. perfringens, C. leptum, C. paraputrificum, Clostridium* sp., and *Eubacterium* sp. using an inhibition assay, ELISA, and Western blotting. The antibody against the *C. perfringens* azoreductase inhibited the azoreductase activities of other bacteria to various degrees. Differences in the inhibition of azoreductase activity among bacterial species could result from differences in antibody–antigen binding or in the degree to which this binding inhibited enzyme activity. In the ELISA assay, the IgG fraction of the antiserum raised against the *C. perfringens* azoreductase cross-reacted with the ammonium sulfate-precipitated antigens in crude extracts from other bacteria tested; the highest reactivity was found with the homologous antigen. In the Western blot, the antibody against the *C. perfringens* azoreductase reacted with the purified azoreductase from each species tested. The immunological cross-reactivity indicates that the azoreductase of *C. perfringens* shares structural similarities with the azoreductases from other species of anaerobic bacteria. These results suggest that the bacterial azoreductases tested may be considered a single related group of enzymes with regard to their function and antigenicity (*11*).

The bacteria isolated from the human intestinal tract that are capable of reducing azo dyes also reduce nitroaromatic compounds. Menadione and *o*-iodosobenzoic acid, which inhibited azoreductase activity, also inhibited nitroreductase activity to the same degree. This was determined by the amounts of reduction of both an azo dye, Direct Blue 15, and 4-nitrobenzoic acid in the presence and absence of each inhibitor (Table 1). FAD enhanced the activity of both azoreductase and nitroreductase; as the concentration of FAD increased, the reduction of both Direct Blue 15 and 4-nitrobenzoic acid also increased.

Table 1. Inhibition of the enzymatic reduction of 4-nitrobenzoic acid and Direct Blue 15 by menadione, *o*-iodosobenzoic acid, or antibody to *C. perfringens* azoreductase.

Inhibitor or antibody	Percent inhibition of reduction	
	Direct Blue 15	4-Nitrobenzoic acid
Menadione[a]	93 ± 7.6	88 ± 17
o-Iodosobenzoic acid[a]	94 ± 8.5	88 ± 17
Azoreductase antibody	72	72

[a]The concentration of menadione or *o*-iodosobenzoic acid was 0.32 mg/ml.

The electrophoretic mobilities of both enzymes from *C. perfringens* were the same on a nondenaturing polyacrylamide gel, as indicated by activity staining for azoreductase and nitroreductase activity. This shows that the combination of size and charge of each of these two proteins was the same (*12*). The antibody against azoreductase inhibited the activities of both azoreductase and nitroreductase (Table 2). It also reacted with gel-purified azoreductase and nitroreductase on a Western blot, suggesting substantial homology between these enzymes. Three nitroaromatic compounds, 4-nitrobenzoic acid, 1-nitropyrene, and 1-amino-7-nitrofluorene, decreased the rate of reduction of Direct Blue 15 by azoreductase (in comparison to the control) from *C. perfringens,* indicating they were substrates for the same enzyme (Table 2). Nitrobenzoic acid was the most inhibitory, followed by 1-nitropyrene and 1-amino-7-nitrofluorene. The kinetics of the inhibition of reduction, using a Lineweaver–Burk plot of Direct Blue 15 reduction, demonstrated competitive inhibition of the reduction by 1-nitropyrene. The apparent K_m for Direct Blue 15 reduction increased in the presence of 1-nitropyrene, with varied concentrations of both Direct Blue 15 and FAD. The pronounced inhibition of the rate of reduction of the azo dye by 1-nitropyrene suggests similarity of the active site of the enzyme for both types of compounds. Taken together, the combined results of immunological, electrophoretic, activation, and inhibition assays demonstrate that the azoreductase in *C. perfringens* reduces both nitroaromatic compounds and azo dyes by a common catalytic site (*12*).

It is likely that the mode of action of the azoreductase from *C. perfringens* is the same as that of the enzymes from *Streptococcus faecalis* and rat liver and that the proteins of the same electrophoretic mobility reduce FAD, which in turn reduces either the azo dye or nitroaromatic compound (*13,14*).

Some of the enzymes involved in electron transfer in anaerobic bacteria that are capable of reducing azo dyes and nitroaromatic compounds may function as azoreductases and nitroreductases. This can be demonstrated on activity-stained anaerobic gels for the detection of azoreductase and dehydrogenase by comparison of electrophoretic mobilities of these enzymes. Crude extracts of several *Clostridium* species were electrophoresed in parallel on a gel. One portion of the gel was stained with nitroblue tetrazolium for the detection of dehydrogenase activity and a second portion of the gel was stained with an

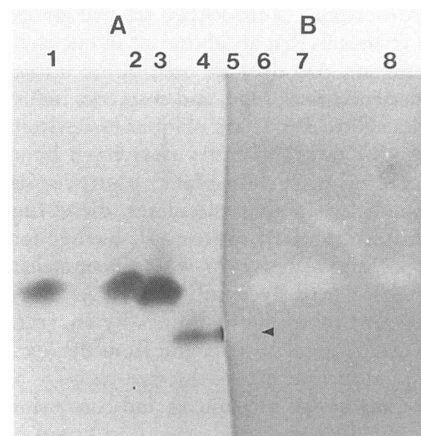

Figure 1. Activity staining of polyacrylamide gel for the detection of dehydrogenases and azoreductases. (*A*) Gel stained with nitroblue tetrazolium for the detection of dehydrogenase. (*B*) Gel stained with azo dye for the detection of azoreductase. Lanes 1,8: *C. paraputrificum.* Lanes 2,3,6,7: *Clostridium* sp. (2 strains). Lanes 4,5: *Clostridium perfringens* (arrow).

Table 2. Effects of nitroaromatic compounds on the reduction of Direct Blue 15 by *C. perfringens.*

Compounds	Reduction of Direct Blue 15, %	Inhibition of dye reduction, %
Direct Blue 15	76.3	0.0
Direct Blue 15 and 4-nitrobenzoic acid	12.2	64.1
Direct Blue 15 and 1-amino-7-nitrofluorene	33.3	43.0
Direct Blue 15 and 1-nitropyrene	36.4	39.9

azo dye, Nitro Red, for the detection of azoreductase. Each of the azoreductase bands had the same mobility as the corresponding dehydrogenase band (Figure 1). In one isolate, two different dehydrogenase bands were present, but only the more rapidly moving bands co-migrated with azoreductase (*7*). In a similar experiment, using 4-nitrobenzoic acid to detect the location of nitroreductase on the gel, the nitroreductase from each of the bacteria also co-migrated with the corresponding dehydrogenase (unpublished data). The results of these experiments provide further evidence that azoreductase and nitroreductase activities may be associated with one protein; these results also indicate that some dehydrogenases possess azoreductase and nitroreductase activity. Although the primary role of the dehydrogenases in anaerobic bacteria is in cellular electron transfer, they may also transfer electrons to xenobiotics similar to azo dyes and nitro-PAHs that may act as nonspecific electron acceptors.

Immunoelectron microscopy, using ultrathin sections of *C. perfringens* cells stained with either preimmune serum or antibody against *C. perfringens* azoreductase and protein A gold, detected the intracellular distribution of azoreductases. Gold particles were dispersed in the cytoplasm with no association with any membranes or other organized structures. There was no aggregation of gold particles, which would show local accumulation of the enzyme before secretion, so the enzyme appears to be secreted as it is synthesized (*15*).

In summary, several anaerobic human intestinal bacteria reduce both azo dyes and nitro-PAHs. Different forms of these enzymes are present in different bacterial genera, but these different forms have structural and immunological homology. Azoreductase and nitroreductase from *C. perfringens* could not be separated by electrophoretical, immunological, or biochemical characteristics. Some dehydrogenases that are involved in cellular electron transfer in anaerobic bacteria may also have azoreductase and nitroreductase activity. Azoreductase is synthesized through the cytoplasm and does not accumulate prior to secretion.

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