Reduction of azo dyes by flavin reductase from *Citrobacter freundii* A1

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Abstract

Citrobacter freundii A1 isolated from a sewage treatment facility was demonstrated to be able to effectively decolorize azo dyes as pure and mixed culture. This study reports on the investigation on the enzymatic systems involved. An assay performed suggested the possible involvement of flavin reductase (Fre) as an azo reductase. A heterologously-expressed recombinant Fre from *C. freundii* A1 was used to investigate its involvement in the azo reduction process. Three model dyes were used, namely Acid Red 27 (AR27), Direct Blue 15 (DB15) and Reactive Black 5 (RB5). AR27 was found to be reduced the fastest by Fre, followed by RB5, and lastly DB15. Redox mediators nicotinamide adenine dinucleotide (NADH) and riboflavin enhance the reduction, suggesting the redox activity of the enzyme. The rate and extent of reduction of the model dyes correlate well with the reduction potentials (E_p). The data presented here strongly suggest that Fre is one of the enzymes responsible for azo reduction in *C. freundii* A1, acting via an oxidation-reduction reaction.

Introduction

Azo dyes are known to pose hazards to human health and the environment, as these dyes do not occur naturally. Thus, all the industrially-produced azo dyes are classified as xenobiotics.¹ Various treatment methods have been used to obviate the problems posed by these dyes and their derivatives. These include physical, chemical and biolog-

ical methods.^{2,4} Among the biological methods used is enzymatic approach, and several enzymes have been reported to degrade azo dyes, including peroxidase,⁵ nitric oxide synthase,⁶ laccase,⁴ and azo reductase.⁷

The enzyme NAD(P)H:flavin oxidoreductase or flavin reductase (Fre) evolved as non-enzymatic reduction of free flavins by nicotinamide adenine dinucleotide phosphate and nicotinamide adenine dinucleotide (NADH) is rather slow and requires organism to possess a system to catalyze the reaction.⁸ Fre catalyzes the reduction of various flavins, namely riboflavin, flavin mononucleotide and flavin adenine dinucleotide (FAD) at the expense of reduced pyridine nucleotides.⁹ Fre from *Sphingomonas* sp. has been shown to possess an azo reductase activity, with the cell extract showing higher activity compared to the whole-cells.¹⁰ One reason being that the cell membranes limit the uptake of highly polar sulfonated azo compounds. Fre could act as an azo reductase under both aerobic and anaerobic conditions.¹⁰ Due to this, it was hypothesized that Fre could act as one of the azo reductases in the biodegradation of azo compounds by *C. freundii* A1. There have been no previous reports on Fre as azo reductase in *C. freundii* to our knowledge.

The potential use of bacterial culture (pure or mixed) to treat dye-containing wastewater has been widely researched, with various bacterial strains reported to be able to decolorize azo dyes. These include several methanogenic bacteria, *Pseudomonas* sp., *Aeromonas* sp., *Bacillus* sp., *Citrobacter* sp., and *Sphingomonas* sp.^{7,11-13} under anaerobic, anoxic and aerobic conditions. Recently, we reported on the capability of a novel NAR-2 bacterial consortium, consisting of *Citrobacter freundii* A1, *Enterococcus casseliflavus* C1 and *Enterobacter cloacae* L17, able to decolorize Amaranth (Acid Red 27) within 30 minutes under microaerophilic condition.¹⁴ The fate of Amaranth degradation by the consortium has also been explored in detail in the report which had led to the proposal of its biodegradation pathways.¹⁴ These unprecedented observations suggested that a thorough research on the mechanism of azo dye degradation and mineralization by the consortium is necessary for future applications, including all the enzymatic systems potentially involved in each strain.

We present here the attempt to characterize an enzyme system in *C. freundii* A1, which relates closely to the ability of the bacterium to decolorize azo dyes in both pure and mixed cultures. The first enzymatic system discovered which relates to its ability to decolorize in mixed culture is a precursor to the synthesis of autoinducer-2.¹⁵ This suggested the role of autoinducer-2 synthase

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(*luxS*) gene product in the decolorization process.¹⁵ Strain A1 was originally isolated from a sewage oxidation pond and has been shown to be able to decolorize azo dyes even as pure culture.^{14,15} Recently, the draft genome sequence of this bacterium has been published, which further assists on the investigation of the decolorization mechanism.¹⁶

This paper describes an investigation on the cellular enzymatic system involved in the azo dye decolorization by *C. freundii* A1, one of the bacteria used in NAR-2 consortium,¹⁴ in order to further understand the mechanism. It also addresses the question: is Fre one of the azo reductases of *C. freundii* A1? The model dyes used in this study are Acid Red 27 (AR27, Amaranth), Reactive Black 5 (RB5, Remazol Black B) and Direct Blue 15 (DB15) (Figure 1), all of which are industrially important dyes. This research, which constitutes part of a bigger study on the mechanism of azo dye degradation by the NAR-2 consortium, is necessary for a more

effective method to treat dye-related wastewater.

Materials and Methods

Preparation of cell extracts, azoreductase and flavin reductase assays

Fresh overnight culture of *C. freundii* A1 in P5 medium (K_2HPO_4 35.2 g/L, KH_2PO_4 20.9 g/L, NH_4Cl 2 g/L, glucose 10 g/L, nutrient broth 2% (w/v), trace elements, pH 7.0) was harvested by centrifugation, and washed 2-3 times in cold potassium phosphate buffer (50 mM, pH 7.5). The supernatant was kept at 4°C prior to detection of extracellular activity. The cells were first re-suspended in the same potassium phosphate buffer, and were maintained as resting cell. The remaining cell suspension was sonicated for a total of 2 min, with 2 min-interval after each 15-S treatment. The cells were incubated on ice during the sonication, using Vibra Cell™ ultrasonic processor (Sonics & Materials, Newtown, CT, USA) using 600 W power output at 0% amplitude. The homogenate was centrifuged at 5000 rpm at 4°C for 30 min. The supernatant was the crude cell-free extract while the pellet contained the cell debris. Protein concentration was determined by Lowry method with bovine serum albumin as standard.¹⁷ The absorbance was read at 750 nm using Cary 100 UV-Visible Spectrophotometer (Varian).

The azoreductase activity of *C. freundii* A1 was detected and measured using a modified procedure.¹⁸ The cell extract (150 µL) was added to a sparged anaerobic solution containing Tris-HCl buffer (50 mM, pH 7.5), Acid Red 27 (0.03 mM) and FAD (0.05 mM). The reaction mix was flushed with nitrogen gas to create an anaerobic condition. The reaction (1 mL) was initiated by the addition of NADH (0.2 mM), followed by 10 min incubation. Reaction progression was monitored using Cary 100 UV-Visible Spectrophotometer (Varian) at the AR27 λ_{max} (521 nm). The azo reductase activity was calculated from the decrease in absorbance, corresponding to the reduction of azo dyes by the enzyme. One unit (U) of the enzyme is defined as the amount catalyzing the reduction of 1 nmol of azo dye per min. The specific activity is defined as units per mg protein. Flavin reductase assay was performed as previously reported by Chan *et al.*¹⁹

Over expression and protein extraction

Over expression and extraction of recombinant Fre was performed as reported previously.¹⁹ Briefly, Fre was expressed in recombinant

E. coli BL21(DE3)pLysS. Upon IPTG induction, it was grown for further 4 h at 37°C. The harvested cells were washed and lysed by sonication. As the recombinant Fre was expressed as inclusion bodies, it was first solubilized and refolded using the Protein Refolding Kit (Novagen, EMD Chemicals, Gibbstown, NJ, USA), and purified using nickel affinity chromatography. Protein concentration was determined using the Lowry assay,¹⁷ and analyzed on 15% SDS-PAGE.

Reduction of azo dyes by Fre

One hundred and fifty µL of the recombinant enzyme was added to the reaction mixture with Tris-HCl (20 mM, pH 7.5), NADH (0.2 mM), riboflavin (15 µM) and the respective azo compounds at a final concentration of 0.1 mM. Reduction rate was determined at 30°C from the decrease in UV-vis absorbance at λ_{max} of each of the dyes (AR27 at 521 nm, DB15 at 585 nm, RB5 at 596 nm) over the period of 30 min. Experiments were performed in several repeats and the best results were plotted. Dye concentrations were determined from the standard curves plotted. Percentage (extent) and rate of dye reduction by Fre were extracted from the plots.

Voltammetric analysis

Voltammetric method involves the application of a variable potential difference between a reference electrode, and a microelectrode (working electrode) at which an electrochemical reaction is induced ($Ox + ne \rightarrow Red$). 'Ox'

and 'Red' represent the oxidized and reduced species respectively, while n is the number of electron. As the potential at the working electrode reaches a value such that a species present in solution is either reduced or oxidized, the current in the circuit increases. This phenomenon is depicted in a *voltammogram*, which is essentially a current-voltage curve, $I=f(E)$, that corresponds to a voltage scan over a range that induces oxidation or reduction of the analytes.²⁰ The voltammetric technique used in this study is the *stripping voltammetry*. This technique was chosen due to its high sensitivity and is among the most widely used voltammetric techniques when analyzing dyes and azo dyes.²¹⁻²³ In stripping voltammetry, a fraction of the electroactive analyte present in the sample is deposited by electrolysis on an electrode in a stirred solution held at a constant potential (pre-concentration stage). Then the stirring is terminated and the potential difference between the working and reference electrode determines the change in potential applied to the working electrode. The analyte is re-dissolved (the stripping stage) by sweeping the potential negatively (cathodic stripping) or positively (anodic stripping).²⁰ The reduction or oxidation of analytes can be measured as current surge and represented as a peak in a voltammogram. An increased sensitivity and better distinction between analytes can be obtained using a *differential pulse* voltage during the stripping stage. In cathodic stripping voltammetry, the more negative the reduction potential, the more difficult it is for the analyte to be reduced.

Figure 1. Structures of the azo dyes used in this study. A) Acid Red 27 (AR27), B) Reactive Black 5 (RB5), C) Direct Blue 15 (DB15).

Cyclic and differential pulse stripping voltammetry was performed using Autolab PGSTAT30 (EcoChemie, Utrecht, The Netherlands) connected to a VA 663 stand (Metrohm, Herisau, Switzerland) at the scan rate of 0.2 V sec^{-1} . Glassy carbon electrode was used as the working electrode completed by means of Ag/Au auxiliary electrode filled with 3 M KCl and Pt electrode as the reference electrode. The experiments were performed in 0.04 M Britton-Robinson buffer (BRb) pH 7 at dye concentrations ranging from 0.001 to 0.1 mM. Dye stock solutions were prepared at the concentration of 1 mM in deionized water. All the solutions were purged with nitrogen gas for 300 s to obtain anaerobic condition prior to determination and thereafter, a blanket of nitrogen gas was maintained throughout the experiment. The buffer and sample mixture was further degassed for 30 s before each measurement was made.

Results

The azo reductase and flavin reductase activities of *C. freundii* A1 cell extracts

The azo reductase activity of various cell extracts is shown in Figure 2. The azo reductase was found to have an intracellular localization, as can be seen from the high specific activity in the cell-free extract. Fre assay was also performed on all the cell fractions (Figure 2) to find out the possibility of it being one of the azo reductases in *C. freundii* A1. It was found that the cell-free extract possesses the highest Fre activity.

Reduction of azo dyes by the recombinant Fre

A recombinant Fre, cloned and purified as described previously,¹⁹ was reacted with three model dyes with different structural complexity. Figure 3A shows the reduction of AR27 by Fre, monitored at its maximum absorbance wavelength (λ_{max}). The absorbance at 340 nm (A_{340}) was also monitored simultaneously to investigate the effects of AR27 reduction on NADH concentration. NADH is an electron donor used in many enzymatic systems. Decrease in A_{340} signifies the oxidation of NADH to NAD^+ ,²⁴ suggesting a continuous utilization of NADH in the process. We observed that the oxidation of NADH ended simultaneously with AR27 reduction. This is probably due to the complete consumption of riboflavin (an electron acceptor), which acts by accepting electron from a redox enzyme and transferring it to the substrate. In order to further confirm that Fre isolated from *C. freundii* A1 is a redox

enzyme, and is critical in azo dye reduction, we then tested the requirements for both NADH and/or riboflavin for AR27 reduction. The result is shown in Figure 3b. The presence of both NADH and riboflavin was found to significantly increase the reduction rate of the dye. AR27 is reduced at approximately $1.15\% \text{ min}^{-1}$ in the first 20 min (estimated using the tangent method), compared to $0.60\% \text{ min}^{-1}$ in the presence of NADH alone. NADH was found to contribute to reduction, even without

riboflavin, due to the direct non-enzymatic reaction. The reduction of RB5 and DB15 were also investigated, and the results are summarized in Table 1. Reduction behavior is similar in the cases of allother model dyes (*data not shown*).

Voltammetric analysis of the azo dyes

Voltammetric measurement was employed to determine the azo bond reduction potentials

Figure 2. The azo reductase and flavin reductase activities of different cell fractions of *C. freundii* A1.

Figure 3. A) Reduction of AR27 by Fre and the simultaneous oxidation of nicotinamide adenine dinucleotide (NADH); B) Reduction of AR27 by Fre in the presence of NADH and/or riboflavin.

(E_p) of the azo dyes to further support any involvements of an electron transfer system. The non-corrected dye reduction potentials obtained are summarized in Table 1. The E_p determined was based on the Ag/AgCl reference electrode, and can be used as is. Potentials at several pHs were initially determined, but the data at pH 7 were deliberately chosen as they closely resemble the pH of the buffer solution in the enzymatic reaction vessel. From Table 1, AR27 is found to be reduced at the lowest (least negative) E_p , followed by RB5, then DB15. As described in Materials and Methods, the more negative E_p signifies the more difficult it is for the azo bond to receive electron(s) to be reduced electrochemically.

Discussion

C. freundii A1 has recently been shown to be able to decolorize azo dye Amaranth (AR27) efficiently as part of a three-bacteria consortium NAR-2.¹⁴ We have also shown before, that it can decolorize even as pure culture but at a slower rate.¹⁵ This prompted us to further investigate the decolorization mechanism, including the enzymatic systems involved, for future applications in dye-containing wastewater treatment. The presence of this xenobiotic compound in wastewater is not only aesthetically unpleasant, but also poses hazards to aquatic microflora due to the toxic reduction products.

The ability of *C. freundii* A1 to decolorize azo dyes may be due to the presence of azoreductase(s), which reduces the azo bond to give amine-containing products. It has also been reported that under anaerobic condition, unspecific cytoplasmic azoreductases would act via the intermediate formation of free reduced soluble flavins.¹⁰ This suggests that flavin reductase (Fre) could be one of the enzymes responsible for azo reduction in *C. freundii* A1. We have previously isolated the *fre* gene, cloned into an expression vector,^{19,25} and the Fre protein was overexpressed in *E. coli*, before being extracted and characterized.¹⁹

In the present study, we investigated the ability of the recombinant enzyme to decolorize azo dyes and also the requirements of cofactors for the enzyme to work *in vitro*. Fre, in general, acts by transferring reducing equivalents or electrons from NADH to flavin (FAD or riboflavin) that serve as redox mediator, followed by the transfer of electrons to the dye molecule. Chemical reaction of the dye with reduced riboflavin is then responsible for the reduction.²⁶ We did not observe any direct reduction by the enzyme itself (data not shown). Reduction is observed immediately upon reaction of the enzyme with an azo dye

(Figure 3). These confirm the azoreductase ability of the recombinant Fre. The redox activity of Fre was then investigated by simultaneously monitoring the NADH oxidation. The observations that NADH is concomitantly oxidized with azo reduction suggest that the cofactor transfers electron to Fre, before being transferred to riboflavin for direct chemical reduction of the azo bonds (Figure 4). The reduction of AR27 in the absence of riboflavin is probably a non-enzymatic reaction by NADH, as reported by others.^{27,28} A general scheme to illustrate the reduction of azo dye in the *C. freundii* Fre system based on our observations is shown in Figure 4. This reaction scheme is classified as indirect (redox mediator catalyzed) biological reduction.²⁹

Voltammetric analysis of the three different dyes used in this study shows that the reducibility of the azo dyes used follows the trend: AR27>RB5>DB15, with AR27 having the highest reducibility while DB15 the lowest. We propose that the difference in reduction rate of the three model dyes is due to the neighbor group effect of the substituents on the dye ring system.^{30,31} AR27 is the simplest to reduce as it is a monoazo dye and the azo bond is sterically less hindered (Figure 1) by the substituents. The different E_p values of RB5 and DB15 may probably be due to i) the structure of DB15, which is more complex compared to RB5; ii) The presence of different substituents at the *ortho*-posi-

tion relative to the azo bonds (Figure 1). This position is occupied by an amino(NH_2)- group in RB5 and a hydroxyl(OH)-group in DB15. The different electronic effects exerted by these substituents onto the ring system might affect the transfer of electron to the respective azo bonds.

It is widely accepted that reduction potential of compounds usually affects the activity of redox enzymes acting on the compound.^{32,33} In our case, we found that both the rate of reduction and extent of reduction (percentage) by Fre of the three dyes tested follow the trend shown by their respective E_p values. AR27 has the highest rate and extent of reduction, and DB15 the lowest. These results strongly support our hypothesis that Fre is the redox enzyme acting as one of the azo reductases in the decolorization mechanism of *C. freundii* A1.

Conclusions

We described here the investigation on the enzymatic system involved in the azo dye biodegradation of *C. freundii* strain A1. Fre was found to be able to act as an azo reductase by reducing aromatic mono- and diazo- dyes in the presence of riboflavin and NADH as electron transfer mediators. The trend of reduction correlates well with the reduction potential, supporting the involvement of an electron

Table 1. Summary of the reduction data of the azo dyes by Fre, and the reduction potentials determined using voltammetry.

Dyes	Rate of reduction* (% min ⁻¹)	Extent of reduction (%) [*]	Reduction potentials (E_p , -V) in BRb pH 7.0
AR27	1.15	26.16	0.514
RB5	0.16	3.53	0.702
DB15	0.06	2.11	0.777

In the presence of both nicotinamide adenine dinucleotide and riboflavin, estimated using the tangent method; ^{}relative to initial concentration, after reaction at 30°C for 30 min. AR27, Acid Red 27; RB5, Reactive Black 5; DB15, Direct Blue 15; BRb, Britton-Robinson buffer.

Figure 4. Proposed scheme depicting the reduction of an azo dye in the Fre system mediated by nicotinamide adenine dinucleotide (NADH) and riboflavin, reproduced (with slight modifications) from Field and Brady (2003), with permission. Dotted line indicates the non-enzymatic reduction by NADH.

transfer system and the redox activity of the enzyme. This further supports the role of Fre as an azo reductase of *C. freundii*. Further research is currently under way to investigate other enzymatic systems that might be involved in the decolorization mechanism, as the possibility of a multi-enzyme system could not be ruled out completely. The findings from this research will assist the understanding of the decolorization mechanisms and pathways in pure and mixed cultures of *C. freundii* A1 for use in wastewater treatment.

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