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Characteristics and purification of an oxygen insensitive azoreductase from *Caulobacter subvibrioides* strain C7-D

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An azo dye-degrading bacterium, *Caulobacter subvibrioides* strain C7-D, semi-constitutively produces an azoreductase that reduced the azo bond of the dyes Acid Orange (AO) 6, AO7, AO8, AO12, Acid Red (AR) 88, AR151, and Methyl Red (MR). This activity was oxygen insensitive. Of the dyes tested, AO7 was the best inducer and the most rapidly reduced substrate suggesting that dye AO7 most closely mimics the natural physiological substrate for this enzyme. The K_m for AO7 was 1 μM. Purification of the azoreductase from *C. subvibrioides* strain C7-D was achieved through dye-ligand affinity chromatography using the dye Orange-A covalently coupled to an agarose support. The azoreductase is approximately 30 kDa and enzyme studies indicate a single azoreductase. The optimal activity, pH, cofactor usage, substrate specificity, molecular weight and K_m characteristics of the enzyme set it apart from other known oxygen-insensitive azoreductases.

Keywords: azo dyes; *Caulobacter subvibriodes*; oxygen-insensitive azoreductase

Introduction

Azo, anthraquinone and triarylmethane synthetic dyes are used for textile dying, paper printing and color photography, with azo dyes representing the largest group of industrial dyes both in number and amount produced [37]. Azo dyes are characterized by a chromophoric azo group represented by the nitrogen to nitrogen double bond (—N=N—). These dyes are not readily degradable in conventional wastewater treatment facilities [1,2]. Furthermore, azo dyes have been reported to be inhibitory to respiration of microorganisms in activated sludge [8,27,28]. This dye toxicity reduces the concentration of heterotrophic bacterial populations, thereby decreasing the overall mineralization of organic compounds, including degradation of dyes [23,24]. Incomplete metabolism of azo dyes, particularly under anaerobic conditions, can lead to increased levels of mutagens and carcinogens in the environment [6,8,26].

Though it was generally assumed that azo dyes can be degraded only anaerobically, there have been some recent reports of aerobic degradation of azo dyes [3,10,15,19,29]. Microbial decolorization of azo dyes often involves initial reductive cleavage of the azo linkage [16,17,20,21]. While under anaerobic conditions incomplete metabolism of the resulting metabolites occurs, aerobic microorganisms may further oxidize the reduced products via deamination and/or hydroxylation reactions [16,18]. Though aerobic bacterial degradation of most azo dyes is difficult to achieve [32], some dyes are less recalcitrant in aerobic environments than others [31,34]. Azoreductases isolated from different organisms vary in their activity and specificity [10,11,30,34]. Though there are some reports of oxygen-insensitive azoreductases, there is very little information available on the purification and characterization of these enzymes [3,15,19,21]. The only other purified enzyme responsible for cleavage of the azo bond under aerobic conditions has high substrate specificity [34,35].

In this study the substrate specificity of the azoreductase from *Caulobacter subvibrioides* strain C7-D was investigated, the effect of dye concentration on enzymatic activity was evaluated and the possibility of the existence of more than one azoreductase was explored. Eleven azo dyes (Figure 1) were used to check for inducibility of azoreductase activity. The azoreductase was purified using dye-ligand chromatography. The enzyme was characterized by non-denaturing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and partial amino acid content analysis.

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Materials and methods

Culture maintenance

Bacterial strain *Caulobacter subvibrioides* strain C7-D was isolated from culture C7, a consortium of two very closely related azo dye-degrading strains supplied by Dr JC Loper, University of Cincinnati. C7 was described in earlier reports as a Gram-negative rod most closely related to *Caulobacter subvibrioides* [12]. This strain was originally obtained from a municipal wastewater treatment facility impacted by the dye industry [12]. The cultures were grown under standard conditions at room temperature in liquid or solid media. A modified Hutner's mineral base medium was used with the following wt/v concentrations: 0.1% NH_4Cl, 0.27% KH_2PO_4, 0.2% Na_2HPO_4, 0.1% D-glucose, and 0.1% v/v glycerol. To prepare 1 L of medium, 20 ml of Hutner's mix was added to 980 ml of Hutner's mineral base medium. Hutner's mix was prepared by dissolving 10 g nitrilotri-

acetic acid in 800 ml distilled water and neutralized to pH 7.0. Then the following were added: $MgSO_4{\cdot}7H_2O$ 14.45 g, $CaCl_2{\cdot}2H_2O$ 3.33 g, $(NH_4)_6Mo_7O_{24}{\cdot}24H_2O$ 9.25 g, $FeSO_4{\cdot}7H_2O$ 0.099 g, Metals-44 mix 50.0 ml, distilled H_2O to 1000 ml. The final pH was adjusted to 7.0. Metals-44 mix contained EDTA 150.0 mg, $ZnSO_4{\cdot}7H_2O$ 1095 mg, $FeSO_4{\cdot}7H_2O$ 500 mg, $MnSO_4{\cdot}H_2O$ 154 mg, $CuSO_4{\cdot}5H_2O$ 39.2 mg, $Co(NO_3)_2{\cdot}6H_2O$ 24.8 mg, $Na_2B_4O_7{\cdot}10H_2O$ 17.7 mg, three drops (0.3 ml) 6 N H_2SO_4 and distilled H_2O to 100 ml. When grown in liquid medium, *C. subvibrioides* C7-D was grown in 250-ml volumes in 2-L flasks shaken at 150 rpm to ensure thorough aeration. This was done to ensure that there was enough head space for the cells to be well aerated. Incubation was terminated at late log phase for preparation of crude cell extracts. Prior to inoculation, strain purity was determined by streaking the culture on minimal medium agar plates containing 80 mg L^{-1} of the dye AO7. Colony morphology and ability to clear the dye were used as indicators of strain purity. Stock cultures were routinely sub-cultured in liquid and solid medium to ensure a steady supply of the strain.

Dyes used

In this study eleven azo dyes were tested (Figure 1) and the common names of the dyes are used as a matter of convenience. The names, abbreviations, Color Index (CI)/Chemical Abstracts Service Registry numbers (CAS), Extinction Coefficients (EC) and their specific Isosbestic Points (IP) are as follows, respectively [13]: Acid Orange 6, AO6, CI 15509, EC 18.5, IP 490 nm; Acid Orange 7, AO7, CI 15510, EC 17.0, IP 483 nm; Acid Orange 8, AO8, CI 15575, EC 15.0, IP 490 nm; Acid Orange 10, AO10, CI 15512, EC 20.5, IP 475 nm; Acid Orange 12, AO12, CI 15970, EC 16.5, IP 482 nm; Acid Orange 52, AO52, CI 13025, EC 42.5, IP 507 nm; Acid Red 27, AR 27, CI 16185, EC 21.9, IP 521 nm, Acid Red 88, AR88, CI 15620, EC 20.1, IP 505 nm; Acid Red 151, AR151, CI 26900, EC 13.7, IP 512; Acid Yellow 9, AY9, CI 13015, EC 13.7, IP 480 nm; Methyl Red, MR, CAS 845–10–3, EC 24.6, IP 408 nm. Azo dyes were obtained from Sigma Chemical Co, St Louis, MO, USA, and further purified by the method of Tabor [22,33]. Dye stock solutions were maintained in phosphate buffered saline (8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 L^{-1} of distilled water). The dyes used in the induction experiments were added to the minimal medium to a concentration of 60 mg L^{-1}. To get a relative value of induction or inhibition, *C. subvibrioides* C7-D cells were also grown without any inducer and the crude extract was assayed for enzymatic activity.

Enzymatic assay

A protein assay based on the method of Bradford was used to determine the protein concentration of the crude cell extracts [4]. The supernatant obtained after centrifuging the cell debris away was used to measure the disappearance of the dye over time The monitored wavelength was the absorption maximum for the particular dye being tested. Phosphate buffered saline solution (PBS) was utilized as an absorbance 'blank'. The reaction mixture consisted of 800 μl of 50 μM dye solution, 100 μl crude cell extract, and 100 μl of freshly prepared 1 mM NADH. The NADH was prepared in 10 mM Tris-HCl buffer (pH 7.2), added to the cuvette and mixed gently to initiate the reaction. Alternatively, freshly prepared 1 mM NADPH was added to the

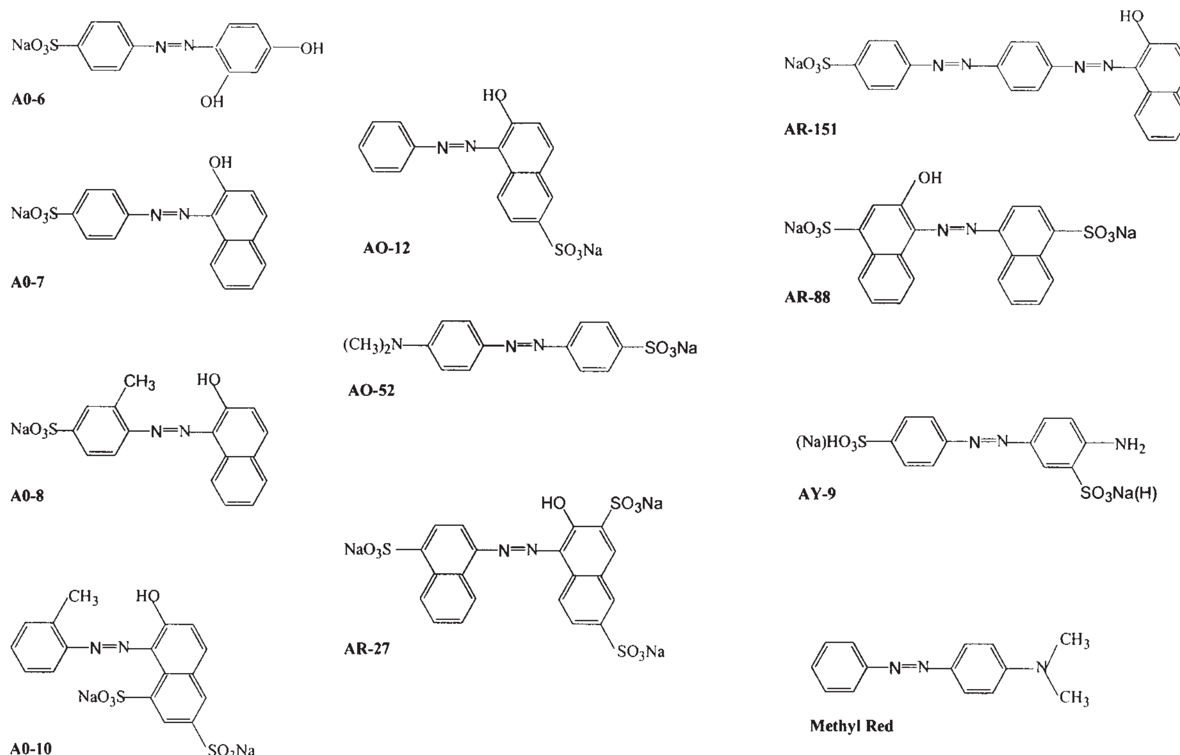

Figure 1 Chemical structures of the azo dyes used.

reaction mixture. FMN could not substitute for NADH or NADPH (data not shown). Change in absorbance of the reaction mixture was monitored for 5 min. One unit (U) of azoreductase activity is defined as the amount yielding reduction of 1 μmol of the dye min^{-1}.

Measurement of effect of induction on enzymatic activity

All steps were carried out under aseptic conditions until the cell pellet was extracted. A loop full of *C. subvibrioides* C7-D (late log or early stationary phase) was added to 5 ml of minimal medium in a 30-ml screw-top test tube. The tubes were shaken on a Burrell wrist action shaker at setting 35 for 24 h at 28°C. The 5-ml broth was then transferred to 240 ml of minimal medium in 2-L flasks, prepared as described above. Filter sterilized azo dye in 5 ml PBS was added after 3 h of incubation to a final concentration of 60 mg L^{-1}. Flasks were incubated with shaking at 150 rpm at 28°C for 36 h. The pellet was washed twice with PBS-EDTA to remove any metals which might hinder enzymatic activity during the enzyme assays. The pellet was again resuspended and washed in PBS to remove traces of EDTA, and the cells were harvested following the above procedure [25]. The supernatant was discarded and the bacterial cell aggregate was re-suspended in 1 ml of PBS and was disrupted by sonication (Braun-Sonic U). The beaker containing the cells was placed in ice during sonication to minimize temperature increase during sonication. Three 15-s bursts at an interval of 5 min at 187 watts and 60 Mhz frequency were sufficient to disrupt the cells. The resultant extract was centrifuged at 20 000 × *g* at 4°C for 60 min. The supernatant was transferred to pre-cooled microcentrifuge tubes with pre-cooled transfer pipettes (Fisher Scientific, Fairlawn, NJ, USA). The microcentrifuge tubes were then centrifuged at 20 000 × g at 4°C for 20 min in an Eppendorf 5415 C microcentrifuge to remove the remaining cell debris. The supernatant was collected for purification procedures and enzyme assays were performed as described above. Michaelis–Menten constants (K_m) were determined from Eadie–Hofstee plots [9]. The K_m was calculated only from the linear reduction rates.

Enzyme purification and analysis

C. subvibrioides C7-D cells were grown in 3 L of modified Hutner's mineral medium containing 60 mg L^{-1} AO7, with shaking till mid-log phase. The cells were harvested by centrifugation, washed three times with 0.1 M sodium phosphate buffer, pH 7.0. The pellet was re-suspended in a minimal volume of buffer and the cells were broken by sonic oscillation as described above. The resulting extract was centrifuged (37 500 × *g*) at 4°C to pellet the cellular debris. The supernatant was transferred to pre-cooled centrifuge tubes and 2% protamine sulfate solution was added to 0.2 ml ml^{-1} of extract with constant stirring. The resultant milky extract was agitated gently in an ice bath for 15 min, after which the extract was centrifuged again at 37 500 × *g* for 20 min. The pellet was discarded and solid ammonium sulfate was added to a concentration of 65% with constant stirring on ice for 15 min. The resulting suspension was then centrifuged at 20 000 × *g* and the supernatant discarded. The pellet was resuspended with a 1:1 volume of sodium phosphate buffer containing 1.0 mM EDTA and 0.1 mM dithiothreitol (DTE).

A fast-performance liquid chromatography (FPLC) apparatus was contained in a refrigerator maintained at 4°C. The extract was passed through an Orange-A (Amicon Corporation, Beverly, MA, USA) column (30 cm × 1.5 cm) previously equilibrated with 50 mM sodium phosphate buffer (pH 7.2). The dyematrix gel in the column was composed of triazinyl dye immobilized on a 6% cross-linked agarose. The flow rate was maintained at 30 ml h^{-1} and 2-ml fractions were collected. Elution was monitored by an in-line UV detector at 254 nm which was connected to a chart recorder. The fractions containing azoreductase activity were pooled. Ultrafiltration (Filtron Corp, Northborough, MA, USA) was performed and fractions were again assayed for activity. Gel electrophoresis under native and denaturing conditions was performed by the method of Zimmerman *et al* [35] with slight modifications. The molecular mass was estimated with markers (10 kDa Protein Ladder; Gibco BRL, Grand Island, NY, USA). For localization of the azoreductase in the gel, activity staining of the gels was performed [35]. The enzyme was run, under native conditions, for 3.5 h at a constant current of 12 mAmp and stained with a solution of 25 μM AO7 in 20 mM HEPES. After staining, the gel was incubated in a solution of 10 mM NADH. Azoreductase activity was shown by clearing of the AO7 dye in the gel. The azoreductase bands from activity gels were excised and treated with 2 ml of an 8 M urea solution for 5 min, after which they were loaded on a nondenaturing gel similar to the previous gel. For amino acid analysis, following electrophoresis, the azoreductase was transferred onto a polyvinylidene difluoride (PVDF) membrane per the manufacturer's instruction (Bio-Rad Laboratories, Hercules, CA, USA). Bands on the PVDF membrane were hydrolyzed in 6 N HCl at 110°C for 18 h, and analyzed with a Beckman 6300 amino acid analyzer. Following SDS-PAGE, gels were stained with Coomassie brilliant blue (Bio-Rad) [25].

Results

Effect of dye in growth medium

Results of the effect of dye in growth medium indicate that enzymatic activity depends on the inducer/inhibitor that was used while growing the cells in minimal medium (Table 1). The decrease in μmol dye min^{-1} g^{-1} of protein × 10 (U g^{-1} × 10) was calculated to compare the rate of dye utilization by *Caulobacter subvibrioides* C7-D azoreductase when induced or inhibited by various azo dyes (Table 1). The fastest reduction of the azo dye AO7 (999 U g^{-1} × 10) occurred when *C. subvibrioides* C7-D was induced by the dye. The slowest reduction of the AO7 occurred when it was induced by AY9 (232 U g^{-1} × 10). A sterile minimal medium solution with dye was used as a control to monitor non-enzymatic azo bond reduction. No such instance occurred in any of the experiments. Activity of the enzyme extracted from *C. subvibrioides* C7-D cells which had not been induced by any dye was assayed (536 U g^{-1} × 10) to get the relative values of induction or inhibition (Table 1). Values greater or smaller than 10% of the azoreductase activity when no dye was present were

Table 1 Substrate specificity and induction patterns of *Caulobacter subvibrioides* strain C7-D azoreductase

Inducer	Specific activity (U g^{-1} of protein) × 10 on:										
	AO6	AO7	AO8	AO10	A012	AO52	AR27	AR88	AR151	AY9	MR
AO6	6	652	125	–[a]	65	–	–	63	80	–	4
AO7	8	999	128	–	91	–	–	163	185	–	4
AO8	8	699	104	–	83	–	–	85	83	–	4
A010	4	571	91	–	48	–	–	48	95	–	7
A012	6	724	177	–	96	–	–	63	154	–	4
A052	6	448	130	–	46	–	–	17	26	–	3
AR27	4	513	157	–	102	–	–	47	41	–	5
AR88	6	732	115	–	92	–	–	92	87	–	4
AR151	4	559	147	–	93	–	–	44	102	–	7
AY9	7	232	70	–	34	–	–	16	15	–	3
MR	4	357	94	–	49	–	–	29	59	–	3
None	2	536	120	–	58	–	–	58	189	–	3

[a]Activity not detected.

arbitrarily considered to be a result of induction or inhibition. Dyes AO6, AO7, AO8, AO12 and AR88 induced azoreductase activity against AO7. There was an inhibition of enzymatic activity against AO7 when AO52, AY9 and MR were present in the culture media. Azo dyes AO10, AR27, and AR151 had no effect on the enzymatic activity against AO7. Comparing the above results with the rate of utilization for other dyes showed that there is a correlation among the different rates of reduction. A decrease or increase in the rate of one dye utilization is proportionately reflected in the degradation rates of the other dyes when the same inducer was used. AR151, however, did not exhibit proportional activity. This difference is speculated to be due to the presence of two azo bonds in AR151.

For cells grown in the presence of AO7, the rate of azoreduction over time was highest with the azo dye AO7 (Figure 2a). For AO8 there was an initial increase then a slow but steady decrease in velocity (Figure 2b). When AO12 was the substrate, the reaction velocity remained constant for a wide range of substrate concentrations (data not shown). Enzymatic activity on AR88 when induced by AO7 was similar to the activity on AO7, except that the increase in velocity was gradual (Figure 2c). When AR151 was the substrate the velocity of the reaction had two peaks presumed to represent the break down of the two azo bonds at different concentrations of the substrate (Figure 2d).

Effect of more than one dye on enzymatic activity

Kinetic studies were conducted by monitoring color disappearance spectrophotometrically. The Eadie–Hofstee plot of velocity against velocity/substrate concentration was used to determine the K_m values [9]. In all experiments K_m was calculated only from linear reduction rates. K_m values of 1, 93, 3 and 4 μM were obtained for AO7, AO8, AR88 and AR151 respectively for C7-D cells grown with AO7 as the inducer in the culture medium (Table 2). Addition of AO8, AR88 or AR151 in equimolar concentrations to AO7 increased the apparent K_m (assayed at the absorption maximum of AO7) of the enzyme for AO7 reduction suggesting competitive inhibition (Table 2). Determination of the K_m value for AO12 was not successful as there was an extended lag period. This could be due to non specific binding of dye to cellular protein, resulting in a fluctuating rate of decolorization over a broad range of substrate concentrations.

Purification of the enzyme

C. subvibrioides C7-D azoreductase was purified 432-fold with a 20% yield using dye-ligand affinity chromatography (Table 3) with an isocratic elution. Active fractions were isolated with 1 M KCl in 20 mM HEPES buffer. The molecular weight of the pure enzyme was established by SDS-PAGE to be approximately 30 kDa.

Denaturing gels of enzyme extracts before and after FPLC were run in order to depict the increase in purification achieved. Azoreductase activity was verified before electrophoresis. The location of the active enzyme was indicated by a clear zone (Figure 3). Activity gel was excised and blotted onto PVDF membrane for amino acid analysis. Activity gels showed the presence of two overlapping bands. The azoreductase could use both NADH and NADPH as cosubstrates. NADH was more efficient in this respect (data not shown). The enzyme had a pH optimum between pH 6.0–7.5.

Figure 3 shows an activity gel and a denaturing gel. Though there appeared to be two overlapping bands in the activity gel, denaturing gels exhibited only one band (Figure 3). The two overlapping bands could be due to differently migrating forms of the same enzyme. The kinetic data in Table 1 also suggest a single azoreductase, corroborating the presence of one azoreductase. Table 4 illustrates the results of a composition analysis of the azoreductase. The azoreductase was rich in hydrophilic residues, with these residues accounting for approximately 57% of the detected amino acids. The major hydrophilic residues are glycine 16%, glutamate and glutamine 16% and aspartate and asparagine 11% of the detected amino acids. Alanine, a hydrophobic residue, comprised approximately 11% of the detected amino acids. Cysteine and tryptophan are artificially listed as 0% because cysteine was not modified for detection and tryptophan was destroyed under the hydrolysis conditions. Proline and methionine were not detected under the assay conditions used.

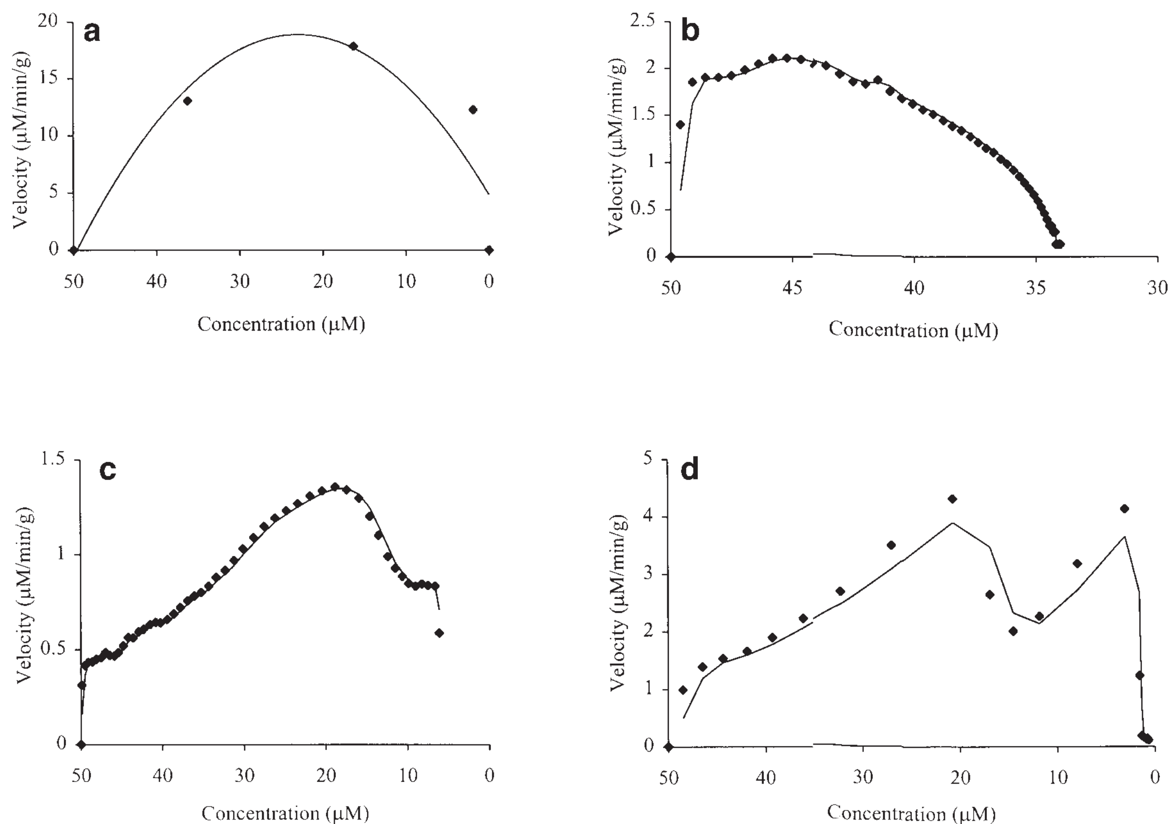

Figure 2 Azoreductase activity on AO7 (a), AO8 (b), AR88 (c) and AR151 (d). Abscissas represent dye concentration. Each data point represents the mean from three different experiments. The assay is described in the text.

Table 2 Apparent K_m values for reduction of the azo dyes by *Caulobacter subvibrioides* C7-D azoreductase. Substrate concentrations are detailed in the text

Substrate	Wavelength (nm)	Inducer	K_m (μM)
AO7	483	AO7	1
AO8	490	AO7	93
AR88	505	AO7	3
AR151	512	AO7	4
AO7	483	none	4
AO8	490	none	450
AR88	505	none	9
A07–AO8	483	AO7	12
AO7–AO8	490	AO7	8
AO7–AO88	483	AO7	3
AO7–AR88	505	AO7	10
AO7–AR151	483	AO7	32
AO7–AR151	512	AO7	34

Table 3 Azoreductase purification summary

Purification step	Total activity (U)	Total protein (g)	Specific activity (U g^{-1})	Yield (%)	Purification (fold)
Crude	79.1	0.49	161	100	1.0
Protamine sulfate	59.8	0.23	260	75	1.6
65% $(NH_4)_2SO_4$	53.2	0.0387	1375	67	8.6
75% $(NH_4)_2SO_4$	45	0.0224	2009	56	12.6
FPLC	16	0.00023	69565	20	432.1

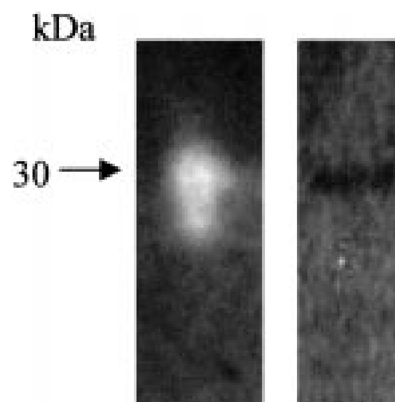

Figure 3 Activity staining of nondenaturing gel (left) and coomassie brilliant blue staining of SDS-PAGE gel (right) of purified *Caulobacter subvibrioides* C7-D azoreductase.

Discussion

The C7-D azoreductase catalyzed the initial step in the oxygen-insensitive reduction of seven of eleven tested azo dyes. Azo dye AO7 was the best inducer indicating that it might be the physiological substrate for the enzyme. Azoreductase activity was cell associated as activity was not detected in the culture supernatants either in the presence or absence of the dyes. The enzyme was inhibited at high AO7 concentrations in the reaction mixture. Azoreductase activity decreased when the concentration of substrate was

Table 4 Amino acid compositional profile of the azoreductase

Name	Mole %	Amount/protein
Asx	11.24	32.331
Thr	4.89	14.057
Ser	7.58	21.806
Glx	15.49	44.576
Pro	0.00	0.000
Gly	15.75	45.331
Ala	11.08	31.890
Val	5.43	15.637
Met	0.00	0.000
Ile	4.80	13.798
Leu	8.53	24.540
Tyr	2.18	6.273
Phe	3.05	9.917
His	2.00	5.756
Lys	2.84	8.182
Arg	4.75	13.672
Cys	0.00	0.000
Trp	0.00	0.000

increased from 50 μM to 250 μM (data not shown). The *C. subvibrioides* C7-D azoreductase was semi-constitutively expressed as shown by activity in the absence of an inducing dye substrate (Table 1). This contrasts with azoreductases purified from *Pseudomonas* sp KF46, *Pseudomonas* sp K24, *Pseudomonas* sp AZR1 and *Klebsiella* sp AZR2, all of which were fully inducible [10,35]. The azoreductase from *Bacillus subtilis* appeared to be synthesized independently of the presence of the dye [36].

Previous research on *Pseudomonas* strains, *Klebsiella* strains, *Bacillus subtilis*, *Shigella dysenteriae*, *Hydrogenophaga palleronii*, *Flavobacterium* sp, *Streptomyces* strains and *Phanerochaete chrysosporium* has described various oxygen-insensitive azoreductases [5,7,14,30,31,34]. The azoreductases are usually substrate-specific and the ease with which these enzymes act on azo bonds depends on the presence or absence of certain functional groups and their position relative to the azo linkage. The azoreductase from *H. palleronii* and *Pseudomonas* K24 cannot degrade Orange II (AO7) but can easily degrade other dyes [3,34]. On the other hand the azoreductase from strain C7-D, *Pseudomonas* K46, or *Shigella dysenteriae* Type 1 can break the azo bond in AO7 [11,35]. Sometimes an organism can have more than one azoreductase which can catalyze the reduction of specific dyes. Azoreductase I but not azoreductase II from *S. dysenteriae* Type 1 acts on Amaranth (AR27) though the other three substrates tested could be reduced by both the azoreductases [11]. Zimmerman *et al* [34,35] studied the intracellular azoreductases and found that the azoreductases of *Pseudomonas* sp were varied and substrate specific. There were aromatic ring substitution patterns which made certain dyes susceptible to enzymatic attack [34,35]. For Orange II azoreductase purified from *Pseudomonas* sp KF46, a hydroxy group in the para position of the napthol ring relative to the azo group, methyl group in the ortho position and electron withdrawing groups on the phenyl ring accelerated the enzymatic attack. A hydroxy group in the ortho position and sulfo and/or phospho groups in proximity to the azo group and a second polar substituent on the dye molecule hindered or completely stopped the enzymatic activity [35]. For Orange I azoreductase purified from *Pseudomonas* sp K24 a hydroxy group in the ortho position was essential for enzymatic action. Substitution of the napthol moiety by an anthranol ring led to a moderate decrease in activity. A hydroxy group in the para position completely stopped enzymatic activity [34]. Work on azo dye-degrading extracellular enzymes from the lignolytic fungus *Phanerochaete chrysosporium* and the bacteria *Streptomyces* sp and *Flavobacterium* sp revealed that extracellular enzymes are also substrate-specific [3,7,11].

In this study we found that the *C. subvibrioides* C7-D azoreductase acted specifically on certain azo compounds. The efficiency with which the enzyme reduced the 11 test azo dyes suggests the structural features required of the substrates by the azoreductase. A hydroxy group at the 2-position of the naphthol ring as in AO7 and AO8 (Figure 1, 2a and 2b) allowed the enzyme to act on the azo bond. Charged groups in proximity to the azo group hindered enzymatic activity (AO10, AO12, AR27 and AY9). Electron withdrawing sulfo (SO^{3-}) groups on the phenyl group appeared to accelerate the reaction (AO7, AO8, AR88 and AR151), and an electron-donating group in proximity to the azo group retarded the reduction (AO10). Sulfo groups apparently had a dual role in its electron withdrawing power as well as a charged group role. AR27 was not reduced as it has three charged (SO^{3-}) groups which had negative contribution towards azo bond reduction. AR88 was reduced as it has only one charged group permitting the azoreductase to act on its azo bond (Figure 2c). AR151 has two azo bonds which were reduced at different concentrations resulting in two distinct peaks (Figure 2d). When the structure had only phenyl rings (AO6, AO52, AY9 and MR), then the electron withdrawing groups did not accelerate the reaction, instead the charged factor of those groups dominated the outcome of the reaction, ie retardation of the enzymatic activity. Azo dyes AO6, AO52 and AY9 were thus not reduced, whereas the azoreductase acted weakly on MR which has no charged substituents on one of its phenyl rings.

FPLC elution results, activity profiles and denaturing gels suggest that there is only one azoreductase present, despite the presence of two bands on activity gels. The enzyme could be present in two confirmations, each with its own migration pattern (Figure 3). This hypothesis is corroborated by the presence of a single band in denaturing SDS-PAGE gels (Figure 3) and the existence of a positive correlation between the rates of specific activity of AO6, AO7, AO8, AO12, AR88 and MR, regardless of which substrate cells were exposed to during growth. Inducibility data further suggest that the *C. subvibrioides* C7-D azoreductase is genetically controlled by a single mechanism.

This research provides an insight into the degradative capabilities of *C. subvibrioides* C7-D. This is believed to be the first report of purification and characterization of an azoreductase from *Caulobacter*. Though the *C. subvibrioides* C7-D 30-kDa azoreductase has approximately the same molecular weight as the Orange II azoreductase purified by Zimmerman *et al* [35], the two azoreductases differ in many respects. The two enzymes differ in their patterns of induction, activity ranges and substrate specificity. The

specificity of induction was much narrower for the Orange II azoreductase that Zimmerman *et al* purified [35] compared to the specificity of the *C. subvibrioides* C7-D inducers. Affinity of the C7-D azoreductase for AO7 ($K_m = 1$ μM) was different from the affinity of the Orange I ($K_m = 2.6$ μM) [34] and Orange II ($K_m = 1.5$ μM) azoreductases toward their primary substrates [35]. With AO8 the K_m values were 93 μM and 1.3 μM for C7-D azoreductase and Orange II azoreductase respectively [35]. An 80-fold increase in the Orange II azoreductase activity was observed when *Pseudomonas* sp KF46 was induced by Orange II (AO7) [35], compared to a 2-fold increase in C7-D azoreductase activity upon induction by the apparent primary substrate. The C7-D azoreductase had a pH optimum between pH 6.0–7.5 which is a narrower range than the 5.0–8.0 optimum of Orange II azoreductase purified by Zimmerman *et al* [35]. The specific activity of C7-D azoreductase when AO7 was the substrate, was 7.8 times higher than the specific activity with AO8, whereas for Orange II azoreductase the difference in activity between the two substrates was only 1.1 times. There was a rise in K_m when AO7 was mixed with AO8, AR88 or AR151 in equimolar amounts (Table 2). This suggests that there was competitive inhibition by substrates AO7–AO8, AO7–AR88, and AO7–AR151.

These data contribute to our understanding of azo dye degradation and suggest the possibility of using azoreductase as part of an at-source wastewater pretreatment system, assuming that cofactor requirements could be met. This pretreatment could remove azo dyes from textile wastewater before it is sent to conventional waste treatment.

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