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Influence of auxochrome group in disperse dyes bearing azo groups as chromophore center in the biotransformation and molecular docking prediction by reductase enzyme: Implications and assessment for environmental toxicity of xenobiotics

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ABSTRACT

Synthetic azo dyes have increasingly become a matter of great concern as a result of the genotoxic and mutagenic potential of the products derived from azo dye biotransformation. This work evaluates the manner in which reducing enzymes produced by *Escherichia coli* (*E. coli*) act on three disperse dyes bearing azo groups, namely Disperse Red 73 (DR 73), Disperse Red 78 (DR 78), and Disperse Red 167 (DR 167). UV–Vis spectrophotometry, high-performance liquid chromatography with diode array detector (HPLC-DAD), and liquid chromatography mass spectrometry (LC-MS/MS) were applied towards the identification of the main products. Seven days of incubation of the azo dyes with the tested enzymes yielded a completely bleached solution. 3–4-Aminophenyl-ethyl-amino-propanitrile was detected following the biotransformation of both DR 73 and DR 78. 4-Nitroaniline and 2-chloro-4-nitroaniline were detected upon the biotransformation of DR 73 and DR 78, respectively. The main products derived from the biotransformation of DR 167 were dimethyl 3,3′-3-acetamido-4-aminophenyl-azanedyl-dipropanoate and 2-chloro-4-nitroaniline. The results imply that DR 73 lost the CN^- substituent during the biotransformation. Furthermore, theoretical calculations were also carried out aiming at evaluating the interaction and reactivity of these compounds with DNA. Taken together, the results indicate that DR 73, DR 78, and DR 167 pose health risks and serious threats to both human beings and the environment at large as their biotransformation produces harmful compounds such as amines, which have been widely condemned by the International Agency for Research on Cancer.

1. Introduction

Annually, hundreds of new colored compounds meant for a multitude of applications in the textile, pharmaceutical, cosmetics, plastics, photographic, automotive, paper and food industries are reported in the literature. The dyes used in the textile industry exhibit a wide range of structures (including azo, chromophore, anthraquinone, arylmethane and indigo groups) and chemical properties (such as solubility and reactive functional groups) (Lewis, 2008). However, the most representative and widely used group belongs to the family of azo dyes, which are characterized by having one or more azo groups (-N=N) attached to aromatic systems (Kunz et al., 2002).

The azo dye structure can be altered through reduction, oxidation (Osugi et al., 2006), acetylation and chlorination reactions (Oliveira et al., 2006). In addition, the dye structure can generate compounds that are even more mutagenic and relatively more harmful to the environment than the dye itself (de Aragão Umbuzeiro et al., 2004). Azo chromophores, which belong to a class of disperse dyes, have drawn a wider attention of researchers (de Aragão Umbuzeiro et al., 2005). Disperse dyes have become a huge problem to the textile industry owing to the fact that they have low solubility in water as this characteristic property favors the loss of 50% dye during the dyeing stage (Golob and Tušek, 1999).

The conventional remediation methods currently employed are incapable of completely degrading the complex structure of these compounds. As a result, azo dyes play a significant role when it comes to the

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toxicological and ecological risks that the discharge of industrial effluents poses to the environment. The release of these improperly treated dyes into nature poses serious threats to aquatic biota and represents considerable health hazards to human beings (Lewis, 1999). Notwithstanding the vast amount of dyes used in the aforementioned range of industries, few studies have, indeed, devoted their attention towards investigating their toxicity. As such, little is known regarding the mutagenic and carcinogenic effects of these compounds. Only a small number of dyes can present acute toxicity. In other words, ingestion of these dyes could lead to immediate death (Rafii et al., 1997).

Considering the difficulties encountered in correlating toxicity and genotoxicity data of azo compounds *in vitro* and *in vivo,* it is essentially important to deepen our studies in that respect so as to devise new mechanisms of elucidation of azo dye carcinogens. In recent years, tests aimed at evaluating the toxicity of xenobiotics have been reported. Among them includes the Ames test which is a bacterial test used for detecting whether compounds under investigation (dyes, for instance) are toxic or not (Brandon et al., 2003) making it a successful tool used for mutagenesis analyses of xenobiotics, since it detects a wide variety of mutagenic compounds and mimetizing the reactions that occur in the intestinal microflora (Novotný et al., 2006).

Franco *et al.* evaluated the mutagenic potential of the DR 73, DR 78 and DR 167 dyes using the Ames test for TA 98 and TA 100 strains. The dyes were analyzed both in the presence of the S9 mixture so as to simulate the biotransformation mediated by CYP isoenzymes and in the absence of exogenous metabolic activation in order to simulate the biotransformation mediated by the intestinal microflora reducing enzymes (azoreductase and nitroreductase) produced by *Salmonella typhimurium.* Disperse Red 73 was considered the most mutagenic dye, considering its mutagenic potential of 30 rev/μg with TA 98 and 34 rev/μg with TA 100 strain, both in the absence of metabolism (-S9). In the presence of S9, the DR 73 presented a reduction in the mutagenic potential after metabolic activation. DR 78 and DR 167 exhibited low mutagenic activity in both the absence and presence of metabolic activity (P-450). Based on the above results, the *Salmonella* / microsome test can be used as an important tool to predict the effects of the ingestion of azo dyes dispersed on human health, clearly indicating that the dyes can be biotransformed (Franco et al., 2018).

The intestine is, indeed, a possible target organ for carcinogenesis after a person is exposed to azo dyes (Sweeney et al., 1994). The intestinal microflora of humans and other animal species can reduce the azo groups of xenobiotics (Cerniglia et al., 1982). However, the specific organisms of the intestinal microflora that take part in azo dye reduction are yet poorly understood (Brown, 1981). The reduction reaction is responsible for the production of aromatic amines, which can act as human carcinogens (Cerniglia et al., 1986; Brüschweiler et al., 2014; Govindwar et al., 2014).

Biotransformation or degradation of an azo dye may occur through a variety of microorganisms, including the aerobic and anaerobic bacteria (Chung and Stevens, 1993). Bacterial degradation of azo dye is usually initiated through the enzymatic biotransformation process with the aid of azoreductase which promotes the cleavage of azo bonds (Zimmermann et al., 1982; Ogawa et al., 1978).

Azoredutase is one of the major reductase type enzymes, which is known to have the ability to metabolize azo dyes ingested by humans into aromatic amines. These aromatic amines are mostly produced by the intestinal microflora or by enzymes present in the cytoplasm of mammalian cells, such as the enzymes belonging to the cytochromes P-450 family (Zanoni et al., 2013).

The docking procedure is clearly a crucial starting point for the commencement of the analysis of substrates and inhibitors in an enzyme active site (Thomsen and Christensen, 2006). This methodology enables us to make predictions regarding the occurrence of near attack conformations (NAC) (Hur and Bruice, 2003), which present the most reactive ground state conformations, deemed to be of great importance. This technique enables one to explore the reactive environment, taking into account the influence of neighboring residues, which could, in principle, modulate the stability of the ligand in the active site, thereby leading to effective reactions (Hur and Bruice, 2003; Luo and Bruice, 2004). In this sense, the molecular docking calculations could be helpful for assessing the influence exerted by substituents in the disperse dyes molecules in the binding modes and interactions with azoredutase active sites.

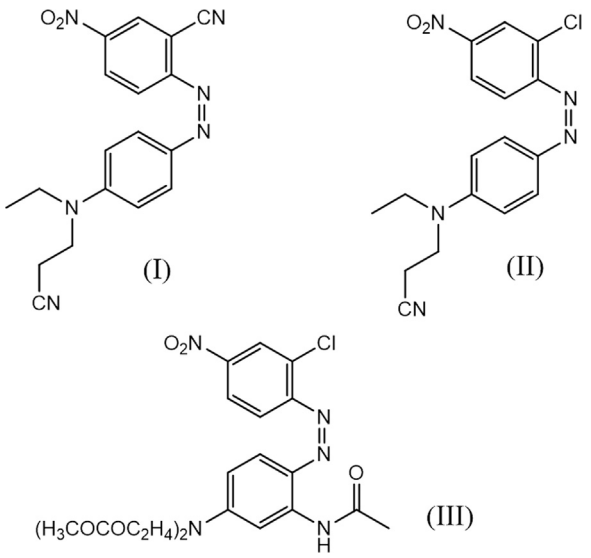

Fig. 1. Structure of Disperse Red 73 (DR 73) (I), Disperse Red 78 (DR 78) (II) and 167 Disperse Red (DR 167) (III) dyes.

Based on the results obtained with the Ames Test by Franco et al., it is extremely important to study the metabolites formed by the reduction of the azo group. Thus, the present work investigates the action mechanism of enzymes present in the intestinal microflora of azo dyes which possess selected substituents including the following: Disperse Red 73, Disperse Red 78 and Disperse Red 167 (chemical structure in Fig. 1). To this end, we investigated the biotransformation products generated by the action of reducing enzymes, such as azoredutase, produced by the anaerobic *Escherichia coli* bacteria. The dyes and their biotransformation products were evaluated by UV–Vis spectrophotometry, high performance liquid chromatography with diode array detector (HPLC-DAD) and mass spectrometry (LC-MS/MS). Furthermore, theoretical calculations were performed so as to confirm the interaction and reactivity of the substituents bonded to the azo group with respect to the azoredutase enzyme. These three dyes were selected for study because of their chemical similarity with only few different ligands. Therefore, we were interested in knowing if azo compounds with similar chemical structures but with few different ligands (CN^- and Cl^-) could provide more or less toxic metabolites after biotransformation process.

2. Experimental

2.1. Materials and reagents

Disperse Red 73 (DR 73), Disperse Red 78 (DR 78), and Disperse Red 167 (DR 167) were obtained from Classic Dyestuffs Inc. Fraction S9 was acquired from Moltox INc. The mineral salts medium (MM) used in all the batch experiments, conducted at pH 7, contained K_2HPO_4 (1.6 g L^{-1}), KH_2PO_4 (0.2 g L^{-1}), $(NH_4)_2SO_4$ (1.0 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.2 g L^{-1}), $FeSO_4{\cdot}7H_2O$ (0.01 g L^{-1}), NaCl (0.1 g L^{-1}), and $CaCl_2{\cdot}2H_2O$ (0.02 g L^{-1}) purchased from Sigma Aldrich. The rich mineral medium (MMR) consisted of MM supplemented with 100 mg L^{-1} of each dye and 1 g L^{-1} of glucose (Sigma Aldrich). The MMR was placed in an autoclave at 121 °C (Himach CR 21 Hitach) for 15 min.