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DYE AFFINITY LABELLING OF YEAST ALCOHOL DEHYDROGENASE

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The interaction of yeast alcohol dehydrogenase (ADH) with the reactive chlorotriazine dye Vilmafix Blue A-R (VBAR) was studied. VBAR was purified to homogeneity on lipophilic Sephadex LH-20 and characterised by reverse phase HPLC and analytical TLC. Incubation of ADH with purified VBAR at pH 8.0 and 37°C resulted in a time-dependent inactivation of the enzyme. The observed rate of enzyme inactivation (k_{obs}) exhibited a non-linear dependence on VBAR concentration from 22 to 106 nmol, with a maximum rate of inactivation (k_3) of 0.134 min^{-1} and k_D of 141.7 μM. The inhibition was irreversible and activity could not be recovered by gel-filtration chromatography. The inactivation of ADH by VBAR was competitively inhibited by the nucleotides NADH and NAD^+. These results suggest that VBAR acts as an affinity label at the nucleotide binding site of yeast ADH.

Keywords: Alcohol dehydrogenase; Affinity labelling; Triazine dyes; Anthraquinone dyes

Abbreviations: ADH, yeast alcohol dehydrogenase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); NAD^+, β-nicotinamide-adenine dinucleotide, oxidised; NADH, β-nicotinamide-adenine dinucleotide, reduced

INTRODUCTION

Reactive triazine dyes have been shown to interact specifically with several proteins and most enzymes recognising nucleotides and nucleotides analogues.[1–6] However, other proteins with no nucleotide binding sites, such as serum albumin, can also bind triazine dyes tightly.[7] Experimental evidence shows that these dyes can bind proteins by both electrostatic/hydrophobic

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interactions preferentially at the substrate binding sites and it is believed that triazine dyes mimic the binding of the naturally occurring anionic coenzymes (e.g. NADH, ATP, coenzyme A).[1–6] Kinetic, difference spectra, molecular modelling and X-ray crystallographic studies have confirmed the similarity between dye and coenzyme binding to their respective complementary enzymes.[1,3,5,6,8,9]

Reactive triazine dyes have been used successfully for the purification and resolution of many proteins by affinity chromatography. From the chromatographic viewpoint, immobilised triazine dyes are preferable to biological ligands since the former are inexpensive, are more resistant to chemical and biological degradation and the degree of purification achieved with triazine dyes is generally better than that obtained by ion-exchange or gel-filtration chromatography.[10–13]

We have previously established the use of the reactive dichlorotriazine dye Vilmafix Blue A-R (VBAR) as a structural probe for labelling the NAD(H) binding site of formate dehydrogenase, malate dehydrogenase and the oxaloacetate binding site of oxaloacetate decarboxylase.[2–6]

Yeast alcohol dehydrogenase (ADH), is a tetrameric enzyme, with an apparent subunit molecular weight of 42 kDa. The enzyme catalyses the interconversion of aldehydes and their corresponding alcohols with the oxidation/reduction of nicotinamide adenine dinucleotide coenzymes NADH and NAD^+. ADH attracts attention because of its usefulness for the determination of ethanol and NAD^+ in food and other biological materials.[14]

The objective of the present work was to study the affinity of the reactive dichlorotriazine dye VBAR for ADH in order to establish the nature of the dye–enzyme interaction and thereby establish a better method for affinity purification of the enzyme based on immobilised dye ligand affinity chromatography.

MATERIALS AND METHODS

Materials

NAD^+ (crystallised, lithium salt, approx. 100%), and crystalline bovine serum albumin (fraction V), were obtained from Boehringer Mannheim, Germany. Yeast ADH, HEPES, and Sephadex LH-20 were obtained from Sigma, USA. VBAR (purity 61.3%, w/w) was a much appreciated gift from Dr. J. Mazza (Vilmax S.A., Buenos Aires, Argentina).

Methods

Assay of Enzyme Activity and Protein

Enzyme assays were performed at 25°C according to a published method[14] using a Hitachi U-2000 double beam UV–VIS spectrophotometer carrying a thermostated cell holder (10 mm pathlength). Activity was deduced by determining the rate of NADH formation from NAD^+ following the increase in absorbance at 340 nm. Protein assays were performed according to a published method[15].

Purification of VBAR

Solid commercial dye (purity 61.3%, w/w) was purified to homogeneity in two stages according to the procedure of Labrou and Clonis[2] which is as follows. One hundred mg dye was dissolved in deionized water (20 ml) by stirring at room temperature. The solution was extracted twice with diethyl ether (2 × 20 ml), the aqueous phase concentrated (*ca.* threefold) on a rotary evaporator and the dye precipitated by addition of cold acetone (60 ml). The precipitate was filtered through Whatman filter paper and dried overnight under reduced pressure. Dried dye was dissolved in water/methanol (5 ml, 50/50, v/v) and filtered through a 0.45-μm-pore-size cellulose membrane filter (Millipore). The dye solution was applied on a lipophilic Sephadex LH-20 column (2.5 × 30 cm) previously equilibrated in water/methanol (50/50, v/v). The column was developed isocratically at a flow rate of 0.1 ml/min/cm. Fractions (5 ml) were collected and analysed by TLC and those with the pure dye were pooled, concentrated by 60% on a rotary evaporator under reduced pressure, before the product was lyophilised and stored desiccated at 4°C.

Analytical Techniques

Analysis of pure dye was performed by TLC and HPLC, whereas dye concentration was determined spectrophotometrically at 620 nm using a molar absorption coefficient[2] of 12.6 l/mmol/cm. Ascending TLC was performed on precoated plastic sheets with silica gel 60 (0.2 mm; Merck) using the solvent system butanol-1/propanol-2/ethyl acetate/water (2/4/1/3). HPLC analysis was effected on a C18 reverse phase S5 ODS2 Spherisorb silica column (250 mm × 4.6 mm ID). The starting solvent system was composed of 80% (v/v) methanol and 20% (v/v) water containing 0.1% (w/v) *N*-cetyltrimethylammonium bromide.

Inactivation of ADH by Vilmafix Blue A-R

Yeast ADH (0.26 units) was incubated at 37°C with various concentrations of VBAR (22–106 nmol) in 1 ml of a solution containing 20 μmol HEPES/NaOH buffer, pH 8.0. The rate of inactivation was followed periodically by removing samples (20 μl) for assay of enzymatic activity. Initial rates of inactivation were deduced from plots of log(% of activity remaining) versus time (min) for several dye concentrations and the slopes and intercepts of secondary double reciprocal plots were calculated by unweighted linear regression analysis.[2-5]

Inactivation studies of ADH by VBAR in the presence of nucleotides (NAD^+ and NADH) were performed in a total volume of 1 ml (37°C) and the reaction mixture contained: HEPES/NaOH buffer pH 8.5 (20 μmol), VBAR (106 nmol), nucleotide (1 μmol) and ADH (0.26 units).

RESULTS

Dye Purification

The reactive dichlorotriazine dye VBAR (Figure 1) was extensively purified from chromophoric, organic and salt impurities that are present in commercial dye preparations and are known to interact with proteins.[2,16,17] Dye was purified to homogeneity in a two step procedure. The preliminary stage was introduced to remove organic-phase soluble contaminants, whereas further purification was achieved by Sephadex LH-20 column chromatography using as a mobile phase water/methanol (50/50, v/v). The degree of purification was assessed by both analytical TLC as well as HPLC. TLC on silica gel plates using as a solvent system butanol-1/propanol-2/ethyl-acetate/water (2 : 4 : 1 : 3) revealed a single blue-absorbing spot with a R_f of 0.69. Analysis of purified dye on RP-HPLC using the ion-pair reagent

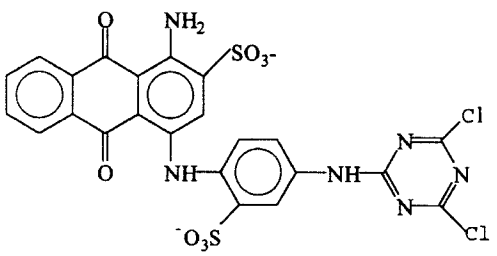

FIGURE 1 The structure of VBAR.

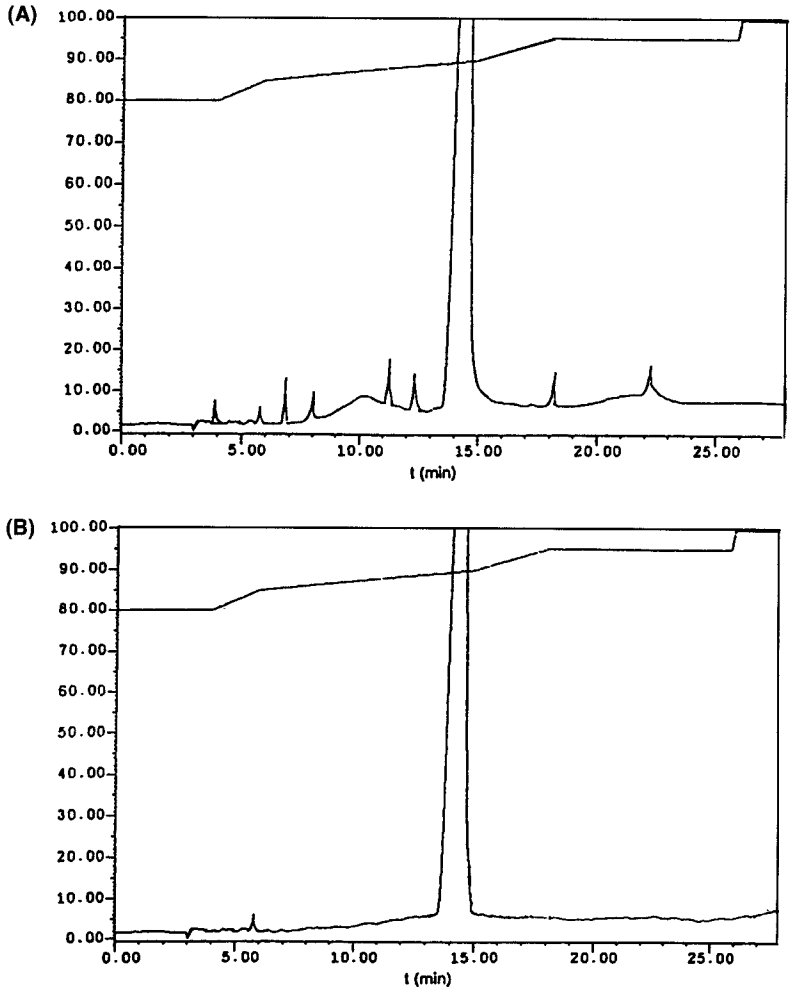

FIGURE 2 Reverse-phase HPLC of purified VBAR. Crude (A), and pure dye (B) were injected on a C18 reverse phase S5 ODS2 Sherisorb silica column (250 × 4.6 mm ID) at a flow rate of 1.0 ml/min under the conditions described in the Methods section. Eluting compounds were monitored at 220 nm.

N-cetyltrimethylammonium bromide showed a single peak (Figure 2). The degree of purity was estimated by peak integration and appeared to be over 98%.

Enzyme Inactivation

Incubation of ADH with VBAR at pH 8.0 resulted in a time-dependent inactivation of the enzyme as shown in Figure 3. The control enzyme,

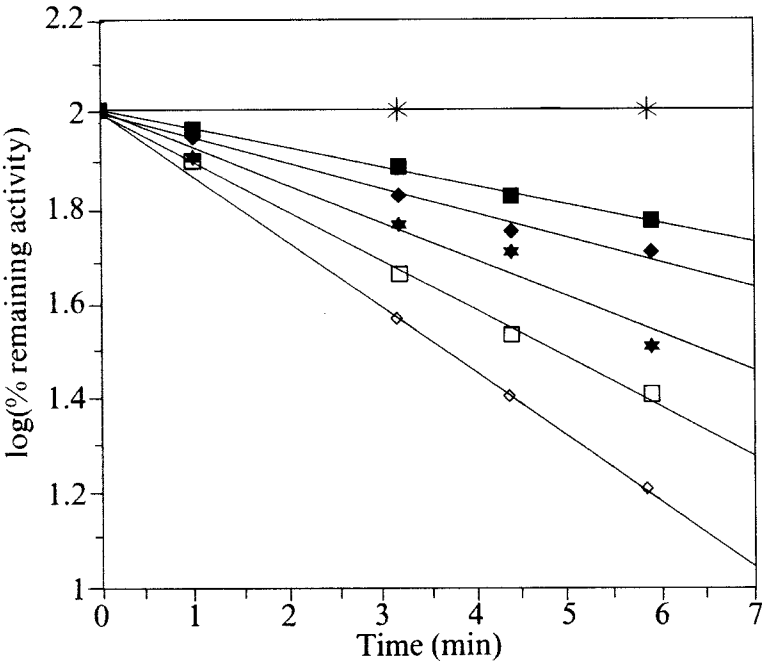

FIGURE 3 Time course for the inactivation of ADH by VBAR at pH 8.0 and 37°C. No VBAR (✳); 22 μM (■); 32.1 μM (◆); 49.8 μM (★); 78 μM (□); 106 μM (◇).

incubated under the same conditions but in the absence of VBAR, showed constant activity over this time period. The enzyme was incubated with various concentrations of VBAR (22–106 μmol) to determine the dependence of the rate of inactivation on the dye concentration. As seen in Figure 3 the observed time of inactivation exhibited logarithmic dependence on the VBAR concentration. Linearity in semilogarithmic plots of the irreversible inactivation process was observed with VBAR concentrations between 22 and 106 μM. The following equation describes the reaction between an active site-directed reagent (e.g. reactive dye D) and an enzyme (E):[1–6,18]

$$1/k_{obs} = 1/k_3 + K_D/(k_3[D]) \quad (1)$$

where k_{obs} is the observed rate of enzyme inactivation for a given concentration of dye D, k_3 is the maximal rate of inactivation (min^{-1}) and K_D is the apparent dissociation constant of the enzyme–dye complex. Regarding ADH inactivation, a plot of $1/k_{obs}$ versus 1/[D] for several concentrations of VBAR (Figure 4) yielded a straight line with a positive ordinate intercept (k_3) of 0.134 min^{-1} and a slope corresponding to an apparent dissociation

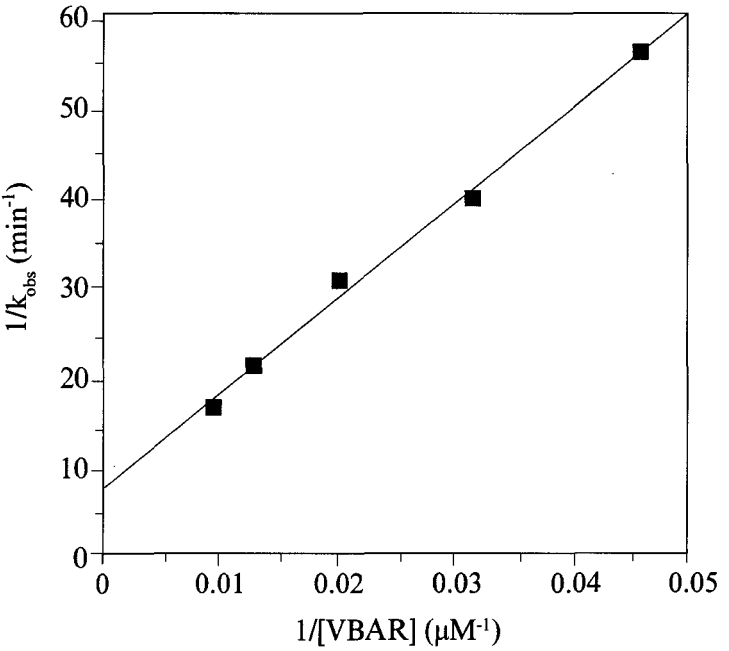

FIGURE 4 Effect of VBAR concentration on the observed rate of inactivation (k_{obs}) of ADH by VBAR expressed as a double-reciprocal plot. VBAR, 22–106 μM. The slope and intercept of the secondary double-reciprocal plot were calculated by unweighted linear regression analysis.

constant of 141.7 μM. In addition inhibition was irreversible since enzyme activity could not be recovered after gel-filtration chromatography on Sephadex G-25. These results are consistent with the conclusion that the dye forms a Michaelis-type reversible complex E–D, before the formation of the covalent product which is rate limiting.

Effect of Competing Nucleotides on the Inactivation Rate of ADH by VBAR

The effect of competing nucleotides (NAD^+, NADH) on the reaction rate of 106 μM VBAR with ADH was investigated and the results are shown in Figure 5. The concentration of the competing nucleotides (1 mM) was chosen to be over 20-fold the K_D reported for NAD^+ and NADH. As shown in Figure 5, NAD^+ and NADH protect yeast ADH against inactivation by VBAR. The protection effect afforded by NADH was more significant compared to NAD^+, at comparable concentrations, in agreement with their relative affinity constants ($K_{m,NAD^+} = 160$ μM, $K_{m,NADH} = 94$ μM).[19]

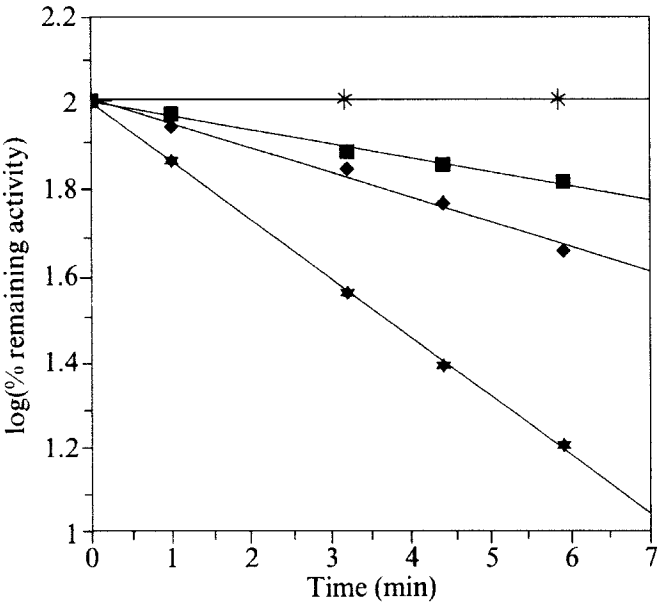

FIGURE 5 Effect of competing nucleotides on the time course for the inactivation of ADH by VBAR (106 μM) at pH 8.0 and 37°C. No VBAR (✳); NADH, 1 mM (■); NAD^+, 1 mM (◆); no addition (✸).

DISCUSSION

The anthraquinone dichlorotriazine dye VBAR was purified on a reverse phase column using *N*-cetyltriethylammonium bromide as an ion-pair reagent. The reverse phase chromatography of dyes with high ionizable polar group (e.g. $-SO_3$) often presents several problems in respect of retention, plate efficiency and peak symmetry.[20] This has been ascribed to a very strong polarity of the $-SO_3$ group, which is able to displace water from silanol sites on the silica surface.[20] The presence of the ion-pair reagent during the chromatography is a valuable factor for improving HPLC separations of dyes since its forms highly stable ion-pairs which do not dissociate in the presence of strongly hydrogen-bonding surface groups. This improves the peak symmetry and the resolution of the chromatography.

In the present study the anthraquinone dichlorotriazine dye VBAR demonstrates the characteristics of an affinity label in its interaction with yeast ADH. It reacts covalently and the observed rate of inactivation of ADH with VBAR exhibits a non-linear dependence on dye concentration, indicating the formation of a reversible enzyme–VBAR complex prior to irreversible modification.[1–6,18]

The ability of specific ligands (substrates and inhibitors, e.g. NADH, NAD^+) to protect enzyme from inactivation by an irreversible inhibitor is usually taken as evidence that the inhibitor is active-site-directed.[2–5] NADH and NAD^+ were tested for their abilities to protect the enzyme against inactivation by 106 μM VBAR at concentrations much higher (20-fold) than their known binding constants. NAD^+ and NADH exhibited significant protection of enzyme from VBAR inactivation. The relative high protection afforded by NADH compared to NAD^+ is in agreement with the relative affinities of the enzyme to the above nucleotides.[19]

Cibacron Blue 3GA (CB3GA) is known to interact with the nucleotide binding domain of horse liver ADH in a manner remarkably similar to the ADP moiety of NAD^+ as revealed by X-ray crystallography.[8] It has been suggested that the anthraquinone ring of CB3GA (Figure 1) binds in a wide apolar cleft of the adenine binding site. The aminobenzene sulfonate ring (Figure 1) is positioned such that its sulfonate ring interacts with the guanidinium site-chain of Arg-271. The triazine ring binds close to where the pyrophosphate moiety of the nicotinamide coenzyme binds. Affinity labelling of horse liver ADH with the reactive chlorotriazine dye Procion Blue MX-R (isomer of VBAR) has shown that the dye behaved as an irreversible active site-directed affinity label and specifically inactivates the enzyme by covalent attachment to the thiol side chain of Cys-174 through nucleophile substitution of the chlorine atom.[9] This amino acid is a key residue in horse liver ADH and lies at the bottom of the active site crevice in the interdomain region of the subunit. Cys-174 as well as Cys-46 and His-67 are the amino acids that ligate the tetrahedrally coordinated Zn^{+2} cation at the enzyme active site.[21] Cys-174 is one of the most conserved residues among ADH and possibly is the same amino acid that is responsible for the nucleophilic reaction with the VBAR in the present study (nucleophilic attack of the cysteinyl –SH group on the carbon–chlorine bond of the VBAR).

Clonis *et al.*,[22] suggested that investigation of dye–protein interaction in solution could provide useful information on which to base new purification protocols. The conditions for specific binding of ADH to VBAR, that was described in this report, could be exploited to design an effective dye–ligand affinity chromatography protocol for ADH purification.

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