Molecular modelling for the design of chimaeric biomimetic dye–ligands and their interaction with bovine heart mitochondrial malate dehydrogenase

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Molecular modelling and kinetic inhibition studies, as well as K_D determinations by both difference-spectra and enzyme-inactivation studies, were employed to assess the ability of purpose-designed chimaeric biomimetic dyes (BM dyes) to act as affinity ligands for bovine heart L-malate dehydrogenase (MDH). Each BM dye was composed of two enzyme-recognition moieties. The terminal biomimetic moiety bore a carboxyl or a keto acid structure linked to the triazine ring, thus mimicking the substrate of MDH. The chromophore anthraquinone moiety remained unchanged and the same as that of the parent dye Vilmax Blue A-R (VBAR), recognizing the nucleotide-binding site of MDH. The monochlorotriazine BM dyes did not inactivate MDH but competitively inhibited inactivation by the parent dichlorotriazine dye VBAR. Dye binding to MDH was accompanied by a characteristic spectral change in the range 500–850 nm. This phenomenon was reversed after titration with increasing amounts of NADH. When compared with VBAR, Cibacron Blue 3GA and two control non-biomimetic anthraquinone dyes, all BM dyes exhibited lower K_D values and therefore higher affinity for

MDH. The enzyme bound preferably to BM ligands substituted with a biomimetic aromatic moiety bearing an α -keto acid group and an amide linkage, rather than a monocarboxyl group. Thus the biomimetic dye bearing *p*-aminobenzyloxanilic acid as its terminal biomimetic moiety (BM5) exhibited the highest affinity (K_D 1.3 μ M, which corresponded to a 219-fold decrease over the K_D of a control dye). BM5 displayed competitive inhibition with respect to both NADH (K_i 2.7 μ M) and oxaloacetate (K_i 9.6 μ M). A combination of molecular modelling and experimental studies has led to certain conclusions. The positioning of the dye in the enzyme is primarily achieved by the recognition and positioning of the nucleotide-pseudomimetic anthraquinone moiety. The hydrophobic groups of the dye provide the driving force for positioning of the ketocarboxyl biomimetic moiety. A match between the alternating polar and hydrophobic regions of the enzyme binding site with those of the biomimetic moiety is desirable. The length of the biomimetic moiety should be conserved in order for the keto acid to approach the enzyme active site and form charge–charge interactions.

INTRODUCTION

The anthraquinone dichlorotriazine dye Vilmax Blue A-R (VBAR) has been shown to act as an affinity label for bovine heart mitochondrial malate dehydrogenase (MDH), inactivating the enzyme in a specific manner via alkylation of an essential lysine residue (the accompanying paper [1]). Furthermore it is known that members of the family of anthraquinone polysulphonated chlorotriazine dyes [e.g. Cibacron Blue 3GA (CB3GA), Procion Blue H-B, Vilmax Blue A-R (VBAR), Procion Blue MX-R] interact with most proteins that recognize nucleotides and nucleotide analogues, often in a manner showing modest selectivity [1a–13]. To tackle this problem, appropriate structural changes of the parent dye (e.g. VBAR) may prove effective, and lead to new purpose-designed biomimetic dyes (BM dyes) with increased affinity for target proteins compared with their commercial anthraquinone counterparts. For example, it may be possible that, if the ability of VBAR to label the nucleotide-binding site of MDH [1] is coupled to suitable substrate-mimetic elements, novel chimaeric biomimetic affinity ligands may be created. These ligands could recognize the coenzyme-binding site (via the anthraquinone/VBAR moiety) and the catalytic site (via the substrate-mimetic moiety) of the target enzyme. In the present work the substrate-mimetic (biomimetic) moiety bears a carboxylated structure – different for every ligand – linked to the reactive chlorotriazine ring, thus mimicking organic acid substrates. This rational approach, when supported by molecular modelling techniques and studied by means of enzyme inactivation, difference-spectral and kinetic

approaches, may lead to new biomimetic tools of increased affinity for MDH. Such biomimetic dye ligands may prove useful materials in enzymological studies and in several affinity-based protein-purification techniques.

EXPERIMENTAL

Materials

NADH (disodium salt; grade II; 98 %), oxaloacetate (free acid) and crystalline BSA (fraction V) were obtained from Boehringer, Mannheim, Germany. Hepes and CB3GA were purchased from Sigma. VBAR and Procion Blue MX-3G (BMX3G) were much appreciated gifts from Dr. J. Mazza (Vilmax S.A., Buenos Aires, Argentina) and Dr. C. R. Lowe (Institute of Biotechnology, University of Cambridge, Cambridge, U.K.) respectively. Bovine heart mitochondrial MDH (specific activity 1300 units/mg) was an in-house product [1].

Model generation

The protein model was built as described by Labrou et al. [1].

The biomimetic dye models were based on the VBAR dye, with different charged terminal biomimetic groups replacing the chlorine atom on the triazine ring. They were built using structural information derived from X-ray-crystallographic structures available from the Cambridge Structural Database [15] and modelled in the binding site as described by Labrou et al. [1]. After positioning the common anthraquinone part of the struc-

Abbreviations used: *Ben*, $-C_6H_4-$ aromatic ring; BM dye, chimaeric biomimetic dye; CB3GA, Cibacron® Blue 3GA; MDH, malate dehydrogenase; VBAR, Vilmax® Blue A-R; VBARCH, VBAR–choline; BMX3G, Procion Blue MX-3G.

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Table 1 Binding characteristics of chimaeric biomimetic dyes BM (1–7), VBAR, CB3GA and control dyes VBARCH and BMX3G to bovine heart MDH.For all dyes X = H, except for BMX3G where one X = SO₃[−] and the other X = H.

Dye–ligand (-R)	K_D (μ M)	
	By inactivation (pH 8.5)	By difference spectra (pH 7.5)
(1) - <i>m</i> -HNBenCOO [−]	6.6 ± 0.5	7.1 ± 0.2
(2) - <i>m</i> -HNBenCH ₂ COO [−]	3.7 ± 0.04	5.5 ± 0.4
(3) -HNCH ₂ COO [−]	20.3 ± 1.60	17.1 ± 0.8
(4) -HN[CH ₂] ₂ COO [−]	24.9 ± 1.80	25.7 ± 1.9
(5) - <i>p</i> -HNBenNHCOCOO [−]	2.0 ± 0.05	1.3 ± 0.03
(6) -SCH ₂ COCOO [−]	6.9 ± 0.70	18.3 ± 1.5
(7) -HN[CH ₂] ₂ NHCOCOO [−]	3.0 ± 0.2	1.8 ± 0.03
(8) - <i>o</i> -HNBenSO ₃ [−] (CB3GA)	4.5 ± 0.30	5.9 ± 0.5
(9) -O[CH ₂] ₂ N ⁺ (CH ₃) ₃ (VBARCH)	—	285 ± 15
(10) -Cl (VBAR)	14.4 ± 1.3	—
(11) -OH (BMX3G)	—	245 ± 16

ture of the parent dye and its biomimetic analogues, the conformation of the biomimetic moiety was investigated in order to avoid bad contacts and optimize hydrogen-bond formation and hydrophobic interactions. In total, eight ligands were modelled and their final conformations optimized by employing molecular graphics and energy-minimization techniques involving the Charm force field *in vacuo* [16]. During energy minimization, the enzyme's amino acids were fixed at their crystallographic positions, but no geometric restraints were applied to the dye analogues.

Synthesis of chimaeric biomimetic dye ligands (Table 1, BM1–7)

Chimaeric biomimetic dye ligands were synthesized, purified and characterized as described by Labrou and Clonis [13]. Choline-VBAR (VBARCH) was synthesized as described [13] with minor modifications. The nucleophilic substitution was carried out at pH 9 using a 20-fold molar excess of choline over VBAR. Unreactive hydrolysed VBAR and BMX3G (i.e. hydroxy-triazinyl dyes) were prepared in 1 % (w/v) Na₂CO₃ (40 °C; 1 h).

MDH assay

Enzyme assays were performed at 25 °C (10 mm pathlength) by a published method [17]. One unit of enzyme activity is defined as the amount that catalyses the conversion of 1 μ mol of oxaloacetate into L-malate in 1 min.

Determination of protein concentration

Protein concentration was determined either by the absorption method (280 nm) using an absorption coefficient ($A_{1\text{cm}}^{1\%}$) of 2.3 for

MDH [18] or by the Lowry method [19] using crystalline BSA (fraction V) as standard.

Enzyme-inactivation studies

Inactivation of MDH by VBAR was performed by the method of Labrou et al. [1]. For the determination of the dissociation constants of the monochlorotriazinyl dyes (BM), the reaction mixture contained in a total volume of 1 ml (35 °C): Hepes/NaOH buffer, pH 8.5, 100 μ mol; VBAR, 0–50.1 nmol; BM dye, typically 20–25 nmol; MDH, 1.3 units.

Difference-spectral titrations

Difference spectra and difference-spectral titrations were performed in a Perkin–Elmer Lambda16 double-beam double monochromator UV-VIS spectrophotometer linked to a Hewlett–Packard ColorPro plotter. The cell holder was thermostatically controlled (25 °C) by a Peltier temperature programmer PTP-1. Enzyme solution (1 ml; 3.0–8.0 nmol of MDH, previously dialysed against 1 litre of 100 mM potassium phosphate, pH 7.5, and clarified through a 0.45 μ m pore size Millipore cellulose membrane filter) and enzyme solvent (1 ml; 100 mM potassium phosphate, pH 7.5) were placed in the sample and reference black-wall silica cuvettes (10 mm pathlength) respectively, and the baseline difference spectrum was recorded in the range 850–500 nm. Identical volumes (2 μ l) of concentrated stock dye solution (0.25 or 0.50 mM) were added to both cuvettes and the difference spectra were recorded after each addition. The maximum difference absorption (peak heights) was measured either relative to a zero-absorbance reference area (830–850 nm) or an isosbestic point. The dissociation constants of monochlorotriazine dyes were deduced from linear-regression analysis of double-reciprocal plots of $1/\Delta A$ versus $1/[\text{dye}]$ [13,20–23].

Dye binding to MDH was reversed by adding NADH (5–10 μ l of a 2 mM solution) stepwise to both cuvettes of the dye-saturated system. Coenzyme addition was stopped after no further absorbance change was observed. Saturation of enzyme with dye in the presence of competing nucleotide ligand (%) was calculated from the expression $(\Delta A/\Delta A_{\text{max}}) \times 100$, where ΔA_{max} corresponded to the value at full saturation.

Kinetic inhibition studies with BM5 dye, hydrolysed VBAR dye and 4-aminobenzoyloxanilic acid

Initial velocities for the MDH-catalysed reaction with NADH as variable substrate were measured in a total assay volume of 3.2 ml containing (25 °C) potassium phosphate buffer, pH 7.5 (100 mM), oxaloacetate (0.23 mM), MDH (0.43 unit) and NADH (13.8–54.6 μ M) in the absence or presence of 0.5, 3.5 or 7.0 μ M BM5 dye, or 0.5, 4.2 or 8.4 μ M hydrolysed VBAR dye, or 0.2, 0.5 or 1 mM 4-aminobenzoyloxanilic acid. With oxaloacetate as variable substrate, the reaction cuvette contained in a total volume of 3.2 ml potassium phosphate buffer, pH 7.5 (100 mM), NADH (0.22 mM), MDH (0.43 unit) and oxaloacetate (15.2–34.5 μ M) in the absence or presence of 0.5, 3.5 or 7.0 μ M BM5 dye, or 0.5, 4.2 or 8.4 μ M hydrolysed VBAR dye, or 0.2, 0.5 or 1 mM 4-aminobenzoyloxanilic acid. The kinetic and inhibition constants were deduced from Lineweaver–Burk plots. Kinetic inhibition studies with BM5 dye, with NADH as a variable substrate, were also performed at various pH values (5.9–8.54) in 100 μ M potassium phosphate buffer. pK_a was determined using the Erithacus software program, obtained from Sigma.

RESULTS

Computer-aided ligand design

The position of the anthraquinone and sulphonate groups

The anthraquinone moiety was placed in the position of the pyrimidine of NAD^+ in a hydrophobic crevice formed by the enzyme's 6–10, 33–36, 76–79 and 94–104 chain regions. The bottom of the cleft is blocked by Tyr-32 and Leu-100 (see Figure 7) so the anthraquinone cannot enter deeper into the crevice. This results in the exposure of the sulphonate and amino groups and one of the carbonyl groups of the ring with all of them pointing towards the solvent, while the carbonyl on the other side of the anthraquinone is placed within hydrogen-bonding distance of Asp-33. An early X-ray-crystallographic study of CB3GA bound to liver alcohol dehydrogenase places the anthraquinone deeper in the crevice, which is unlikely in the case of MDH without changing the conformation of the protein since the crevice is not deep enough to accommodate the polycyclic moiety in the way proposed by Biellmann et al. [7]. The only sulphonate group of the anthraquinone is exposed to the solvent and could form intramolecular hydrogen bonds with the nearby amino group. However, the control dye BMX3G, an analogue of VBAR which bears an additional SO_3^- group on the anthraquinone ring, bound very inefficiently to the enzyme. This is due to the introduction of a negative charge in a negatively charged region of the binding pocket of MDH, around residue Asp-33.

The position of the triazine ring

The triazine ring binds in the region where the coenzyme pyrophosphate binds, with one side close to the surface of the enzyme. This leads to the speculation that possible immobilization of the ligand on a solid support via the triazine ring should not drastically alter its binding to the enzyme. This may be important when exploiting the dye ligands in affinity techniques for protein purification. On the other side of the triazine ring, towards the inner of the binding sites, the substitution point of different biomimetic moieties is located. On seven of the eight analogues tested, an $-\text{NH}-$ group is attached to the triazine ring positioned within hydrogen-bonding distance from the carbonyl of Gly-77 (Figure 7).

The position of the biomimetic moiety

The choice and positioning of the terminal biomimetic moiety of the ligands differs from the experimental study on alcohol dehydrogenase [7]. In the present study the design mimics the cofactor interactions with the enzyme. The biomimetic dyes modelled and tested here are distinguished into having a benzene ring connected to the amino group attached to the triazine ring, and those that have an aliphatic chain instead of the benzene ring. The terminal charged groups present are carboxyl, carboxymethyl (acetate), ketocarboxyl, aminoketocarboxyl (oxamate) and sulphonate. The major structural features of the biomimetic analogues are the length and distribution of the hydrophobic, polar and charged groups. In addition, a choline derivative of VBAR (VBARCh) was tested as a control against the biomimetic moiety attached to the triazine ring of bifunctional biomimetic ligands. The control dye VBARCh binds to MDH very weakly.

The benzene ring is placed in the space occupied by the second ribose ring (linked to the nicotinamide) in the hydrophobic cavity formed by Ile-12, Ile-116, Asn-118, Pro-75 and fragments of the main chain of the enzyme. The methyl and ethyl groups for those analogues that have an aliphatic chain instead of a benzene ring occupy the same cavity, but, of course, they are shorter in

Figure 1 Effect of competing BM5 dye on the time course for the inactivation of MDH by VBAR at pH 8.5 and 35 °C

All incubation mixtures contained 25.0 μM BM5 with the following VBAR concentration (μM): no VBAR (■); 16.4 (◆); 24.6 (○); 32.8 (★); 41.2 (□); 50.1 (*).

length. The terminal carboxylate group is attached to the benzene or methyl/ethyl group either directly or via a methylketo or amide group as a spacer. These are positioned next to the active centre where the nicotinamide of the cofactor is located. In the longer of the analogues (BM5) the carboxylate superimposes readily on the amide group of the nicotinamide moiety whereas the other analogues are shorter. The nicotinamide-binding cavity is fairly polar, and, in the crystal structure of pig MDH [24], several water molecules have been identified. Hydrogen bonds between the donor groups of the analogues ($-\text{NH}-$) and carbonyl groups of the proteins as well as water molecules could be formed. In the case of the control dye VBARCh, the charged bulky choline moiety is placed against the hydrophobic Ile-12 and Ile-117 in a rather restricted volume to accommodate the choline group.

Enzyme-inactivation studies

It has been shown that the dichlorotriazine dye VBAR specifically inactivates bovine heart MDH, and the inactivation process is time- and dye-concentration-dependent, following saturation kinetics [1]. Competitive inhibitors and substrates reduce the observed rate of enzyme inactivation (k_{obs}) by active-site-directed agents (e.g. VBAR) according to the equation:

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

where I is the competitive inhibitor and K_I its apparent dissociation constant [10,12–14,22,25,26]. All the chimaeric biomimetic dyes did not inactivate MDH but behaved as competitive inhibitors with respect to VBAR, since they decelerated the rate of enzyme inactivation by VBAR. Figure 1 shows the events taking place during MDH inactivation (16.4–50.1 μM VBAR) in the presence of BM5 (25 μM). The apparent dissociation constants of monochlorotriazine dyes (Table 1) were calculated from double-reciprocal plots (Figure 2) depicting the apparent rate constants of MDH inactivation versus VBAR concentration, in the presence of monochlorotriazine dye.

Difference-spectroscopic studies

The interaction between dye and MDH was also investigated by studying the perturbation of the absorption spectrum of the dye.

Figure 2 Effect of dye concentration on the observed rate of inactivation (k_{obs}) of MDH by VBAR in the presence of competing BM5 dye

VBAR, 16.4–50.1 μM ; BM5, 25.0 μM . The slopes and intercepts of the secondary double-reciprocal plot were calculated by unweighted linear-regression analysis.

Non-inactivating chimaeric BM dyes and terminal-ring sulphonated CB3GA exhibited marked spectral changes on interacting with binding sites on MDH. The nearly neutral pH employed is the pH required for maximum MDH activity. This probably affords an enzyme conformation similar to that adopted during catalysis, thus creating more favourable conditions for ligand binding. Furthermore, at nearly neutral pH, no covalent binding of BM to MDH occurred and no enzyme inactivation was observed.

Dye–ligand binding to MDH was assessed by difference spectroscopy in the 850–500 nm region. In the presence of MDH the absorption spectrum of the dyes undergoes a red shift, producing difference spectra consisting of a positive maximum in the 670–680 nm region. Figure 3 (top) depicts original difference spectra recorded directly on the plotter. BM5 displays two broad peaks (a 675 nm positive and 540 nm negative) and an isosbestic point at 590 nm, following a shift from its original absorbance maximum (614 nm). The shape and wavelengths corresponding to the maximum and minimum of the dye spectrum remained unchanged during titration experiments, and furthermore no time-dependent changes in absorbance were observed. These findings are indicative [21] of no irreversible dye binding to MDH and formation of one type of complex. The increase in absorbance at positive maximum after each addition of the dye exhibits a hyperbolic dependence on dye concentration, indicating the formation of a dye–MDH complex. This phenomenon was useful for the calculation of the K_D of the BM5–MDH complex, in addition to the K_D values determined by enzyme-inactivation studies. Dissociation constants for all dyes were calculated from double-reciprocal plots of absorbance difference versus dye concentration (Figure 3, bottom). The intercept on the abscissa corresponds to the dissociation constant of the dye–MDH complex [13,21–23,27,28]. BM5 exhibited the lowest K_D value (1.3 μM ; Table 1) with respect to MDH.

The biomimetic dye–MDH complex could be dissociated by the addition of NADH. A solution of enzyme with saturating dye concentration was titrated with nucleotide and the difference spectrum recorded against a dye-blank buffer. A decrease in the ΔA_{max} values was observed with increasing nucleotide concentration, suggesting displacement of the dye from the dye–

Figure 3 Difference spectra of BM5 (from bottom to top: 0.86–3.0 μM) in the presence of MDH (3 μM) at pH 7.5 and 25 °C (top) and difference absorbance at 690 nm as a function of the total BM5 concentration (0.43–3.0 μM) expressed as a double-reciprocal plot (bottom)

The dissociation constants were deduced from linear-regression analysis of the double-reciprocal plots.

Figure 4 Effect of the concentration of competing NADH on the dye–MDH complex at pH 7.5 and 25 °C

Saturation of MDH with dye is expressed as a percentage. $\circ$, BM5 dye; $\blacklozenge$, CB3GA dye.

MDH complex (Figure 4). Dyes exhibiting higher affinity for the enzyme required a larger amount of nucleotide to dissociate the respective dye–enzyme complex. For example, the nucleotide/dye

Figure 5 Lineweaver–Burk plots for the determination of kinetic and inhibition constants

The insets show the replot of slopes of the double-reciprocal plots versus BM5 concentration. Top, inhibition of MDH by BM5 at different NADH concentrations (25 °C). The enzyme was assayed in the absence (+) or presence of BM5 (μM): 0.5 (○); 3.5 (◇); 7.0 (◆). Bottom, inhibition of MDH by BM5 at different oxaloacetate concentrations (25 °C). The enzyme was assayed in the absence (+) or presence of BM5 (μM): 0.5 (○); 3.5 (◇); 7.0 (◆).

Table 2 Kinetic inhibition constants for the inhibition of MDH by BM5, hydrolysed VBAR and *p*-aminobenzoyloxanilic acid

Inhibitor	Substrate varied	Inhibition pattern	K_i (μM)
BM5	NADH	Competitive	2.7
BM5	Oxaloacetate	Competitive	9.6
Hydrolysed VBAR	NADH	Competitive	12.6
Hydrolysed VBAR	Oxaloacetate	Mixed-type	14.5
<i>p</i> -Aminobenzoyloxanilic acid	NADH	Non-competitive	590
<i>p</i> -Aminobenzoyloxanilic acid	Oxaloacetate	Competitive	1160

ratio required to dissociate the respective dye–enzyme complex by 50% was 50 for BM5–MDH complex and 15.8 for CB3GA–MDH.

Figure 6 pH profile for binding of BM5 dye to MDH

NADH was the variable substrate with oxaloacetate at saturating levels. pK_a was determined using the Erithacus software program (Sigma).

Kinetic inhibition studies

Kinetic inhibition studies of bovine heart MDH were performed with BM5 dye (Figure 5), unreactive hydrolysed VBAR dye and 4-aminobenzoyloxanilic acid as inhibitors, and NADH and oxaloacetate as variable substrates. The inhibition patterns and constants obtained are summarized in Table 2. The effect of pH variation (5.9–8.54) on pK_i for BM5 dye versus NADH at saturating concentration of oxaloacetate is shown in Figure 6.

Kinetic inhibition studies of MDH were performed with BM5 dye as inhibitor, and NADH and oxaloacetate as variable substrates. BM5 displayed competitive inhibition with respect to both NADH (K_i 2.7 μM ; Figure 5, top) and oxaloacetate (K_i 9.6 μM ; Figure 5, bottom).

DISCUSSION

Bovine heart MDH requires the α -keto acid oxaloacetate as its natural substrate, and, as it is a nucleotide-dependent biocatalyst, it should recognize anthraquinone dyes as coenzyme pseudo-analogues [1,4–6,9–13,29]. Each chimaeric biomimetic dye is an analogue of the parent dye, VBAR, and exhibits two enzyme-recognition moieties, thus possessing dual functionality. Both carboxyl and keto acid groups were used as biomimetic functions of the chimaeric dyes (Table 1) which were designed to exhibit higher affinity for MDH, compared with the commercial anthraquinone dyes, CB3GA and VBAR. In designing the biomimetic ligands, emphasis was placed on mimicking the coenzyme alternating polar and hydrophobic regions and introducing restriction in flexibility without too much steric hindrance. From both the X-ray-crystallographic study of CB3GA on alcohol dehydrogenase [7] and the binding study of dichloro Procion Blue MX-R (isomer of VBAR) on lactate dehydrogenase [10] it is evident that the triazine ring should be located partly at the exposed region of the binding site near the surface of the enzyme. The reasoning behind the introduction of an NH-group into the biomimetic moiety immediately after the triazine ring, occupying the space of the pyrophosphate of NAD^+ , was to use the proton donor to form a hydrogen bond (Figure 7). The most favourable conformation of the biomimetic dye places the NH- group

Figure 7 For legend see facing page.

within hydrogen-bonding distance of the carbonyl of Gly-77, a hydrogen-bond equivalent of the pyrophosphate oxygen of NAD^+ to the amino group of Ile-12. The next part of the biomimetic moiety, in the direction leading to the active site of the enzyme, was modelled as hydrophobic in order to be accommodated in the hydrophobic cavity formed by Ile-12, Ile-

116, Asn-118 and Pro-75. Two routes were followed. (1) A bulky benzene ring was introduced, with the rest of the biomimetic chain attached at the *para* (BM5) or *meta* (BM1) position (Figure 7). In this way a spacer is added without introducing extra degrees of freedom. In the case of BM5, the direction of the biomimetic moiety is conserved while introducing maximum

Figure 7 Stereodiagrams of the biomimetic dyes BM5, BM1, BM3 and BM7 in the coenzyme-binding site of pig mitochondrial MDH

The diagrams were produced using the program MOLSCRIPT [30].

length. (2) A flexible aliphatic C-1 (BM3) or C-2 (BM7) group was introduced assuming a change of direction and more conformational freedom (Figure 7). Aliphatic groups proved to be less effective in dye binding to MDH than the benzene ring groups. This may result from the fact that the former are shorter in length than the benzene ring analogues, thus bringing the

terminal keto acid group closer to hydrophobic parts of the binding site of MDH.

As a control for the mode of binding, we tested a choline derivative of VBAR (VBARCh), where a bulky positively charged group is introduced as a triazine-linked terminal moiety. This dye together with BMX3G was mainly used to demonstrate that

the purpose-designed bifunctional (chimaeric) biomimetic dyes bind in a way similar to the cofactor. The introduction on the parent dye, VBAR, of groups causing unfavourable interactions with MDH, thus diminishing binding ability, supports this argument.

The positioning of the keto acid of the biomimetic moiety in the active site, where several candidates for charged interactions are available, was modelled to mimic that of the nicotinamide moiety of NAD⁺. This was achieved by introducing aliphatic or amide groups at either the *para* position of the benzene ring or the aliphatic ethyl spacer (Table 1). From the molecular model (Figure 7) it is evident that, without a spacer possessing hydrogen-bonding ability on the terminal benzene or aliphatic C-2 group, the keto acid group cannot approach the polar active site of MDH. To this end, introduction of just a C-1 spacer places the keto acid out of the hydrophobic part of the binding site, and introduction of an amide brings the keto acid nearer the active site. The latter enhances the possibility of additional hydrogen-bond formation with the amide donor and carbonyl acceptor.

The conclusions obtained from the molecular modelling study of biomimetic dyes and MDH were confirmed experimentally. Monochlorotriazine chimaeric biomimetic dyes are markedly less reactive than the corresponding dichloro ligands, and they fail to inactivate MDH. Lower reactivity of monochloro dyes with target enzymes has been reported previously [3,10,13,22,31]. Substitution at the triazine ring of VBAR with aliphatic or aromatic acids and keto acids led to analogues exhibiting affinity higher than that of the parent VBAR and CB3GA dyes. This view is supported by the respective K_D values obtained both by enzyme-inactivation and difference-spectra studies (Table 1). Generally we obtained good agreement, in relative terms, between K_D values measured by the two methods. As expected, absolute K_D values for the same dye differed because of the different experimental conditions. MDH bound preferably to BM dyes that bore aromatic and/or keto acids, incorporating an amide linkage, at the terminal biomimetic moiety. Of these, BM5, which has a terminal aromatic keto acid function with amide linkage, clearly displayed the lowest K_D value. Previous observations confirmed that dyes bind to proteins effectively as the result of their hydrophobic nature and polarized groups which promote ionic interactions [6]. The hydrophobic nature of the binding site of MDH has been demonstrated by Raman spectroscopy studies [32]. These results are in agreement with our observation that aromatic terminal biomimetic moieties (*-Ben*-designation) enhance the strength of dye interaction with MDH (Table 1).

The finding that the addition of natural ligands to the MDH–BM complex (e.g. NAD⁺, NADH) displaced the dye from the complex in a concentration-dependent manner strengthens the view that BM dyes interact with enzymes as nucleotide pseudoanalogues and occupy the nucleotide-binding site. Moreover, those BM dyes displaying higher affinity required higher nucleotide concentration for dissociation of the respective MDH–BM complex. These observations indicate that BM dyes bind to the same site of MDH as the nucleotide. Furthermore kinetic inhibition studies confirmed this view. The dye BM5 exhibited a competitive-type of inhibition with respect to both substrates, NADH and oxaloacetate.

Kinetic inhibition studies with unreactive hydrolysed VBAR, a dye that possesses anthraquinone but no terminal biomimetic moiety, gave different inhibition patterns (Table 2). This dye proved to be a competitive inhibitor with respect to NADH, and a mixed-type inhibitor with respect to oxaloacetate. On the other hand, *p*-aminobenzoyloxanilic acid, the terminal biomimetic moiety of BM5, gave a weak inhibition pattern with MDH

(Table 2), displaying competitive inhibition with respect to oxaloacetate and non-competitive inhibition with respect to NADH. The replacement of a hydroxy group with a *p*-aminobenzoyloxanilic acid group results in a decrease in binding energy equivalent to -3.8 kJ/mol [calculated from the relationship $\Delta G = -RT \ln(K_1^{\text{hydrolysed VBAR}}/K_1^{\text{BM5}})$]. The presence of the blue chromophore on *p*-aminobenzoyloxanilic acid leads to a decrease in binding energy equivalent to -13.3 kJ/mol [calculated from the relationship $\Delta G = -RT \ln(K_1^{\text{p-aminobenzoyloxanilic acid}}/K_1^{\text{BM5}})$].

The inhibition of MDH by BM5 dye is found to be pH-dependent (Figure 6), and the K_i values increased with increasing pH values. Binding of BM5 dye to MDH is prevented when a binding-site group of MDH of pK_a 7.2 is deprotonated (Figure 6), probably a histidine. From the molecular model (Figure 7, BM5) the only histidine available is His-176 of the active site, located in the vicinity of the negatively charged terminal biomimetic moiety. On the basis of X-ray-crystallographic studies it has been proposed that binding of NADH to MDH reduces the solvent accessibility to the active site, thereby causing a reduction in the apparent dielectric constant within this site, resulting in an increase in the apparent pK_a of the His-Asp pair in the active site [33]. Lodola et al. [34] have shown that binding of NADH causes an increase in pK_a from 6.4 to 7.4. The above abnormally high pK_a is at the upper end of the range expected for imidazole dissociation and is a general characteristic of the His-Asp pair found in 2-hydroxy acid dehydrogenases [34,35], serine proteases [36] and thermolysin [37].

From the discussion so far and Table 1, it appears that the biomimetic dyes fall into three categories, on the basis of their structural conformation and measured K_D values: (1) those that possess both a long (benzene or ethyl) spacer and an amide group to bring the keto acid closer to the active site (K_D values 1.3–1.8 μM); (2) those that possess a benzene spacer but do not have the amide group (linkage), and therefore have one or two hydrogen bonds less and are of shorter length (K_D 5.5–7.1 μM); (3) those that have neither (K_D 17.1–25.7 μM). From this classification and the combination of modelling and experimental studies, certain conclusions on the binding of biomimetic dyes to MDH can be drawn. The positioning of the dye is primarily achieved by the recognition and positioning of the nucleotide-pseudomimetic anthraquinone moiety. The hydrophobic groups of the dye provide the driving force for the positioning of the ketocarboxyl-biomimetic moiety. It is important to achieve a match between the alternating polar and hydrophobic regions of the protein binding site with those of the biomimetic moiety. Also, the length of the biomimetic part should be conserved, in order for the keto acid to approach the active site and form the terminal charged interactions at the nicotinamide-binding site.

The biomimetic dyes investigated in the present work are expected to become useful affinity ligands for enzymological studies, and eventually lead to new affinity tools for enzyme purification. For example, when these dyes were immobilized on agarose, the respective affinity adsorbents were successfully used to purify MDH from bovine heart extract [38].

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