

Dye-affinity labelling of bovine heart mitochondrial malate dehydrogenase and study of the NADH-binding site

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The ability of the reactive dichlorotriazine dye Vilmax Blue A-R (VBAR) to act as an affinity label for bovine heart L-malate dehydrogenase (MDH) was studied. VBAR binds specifically and irreversibly to MDH (k_3 0.16 min⁻¹; K_D 14.4 μ M). The inactivation of the NADH-dependent enzyme by VBAR is competitively inhibited by NAD⁺, NADH and ADP. Quantitatively inhibited MDH contained approx. 1 mol of dye per mol of active site. The inhibition is irreversible and activity cannot be recovered either on incubation with 10 mM NAD⁺, 10 mM NADH or 10 mM ADP, or by extensive dialysis or gel-filtration chromatography. Data obtained from high-perform-

ance gel-filtration chromatography and analysed by Scatchard plot suggested the presence of two coenzyme-binding sites per MDH dimer. Tryptic digestion of VBAR-labelled MDH followed by reverse-phase HPLC analysis revealed one VBAR-labelled peptide. It appears that each subunit features the same peptide bearing the modifying residue involved in MDH labelling. The pK_a of the modifying residue is 8.05. Both total acid hydrolysis of VBAR-labelled MDH followed by HPLC and TLC analysis, and molecular-modelling studies suggest that the modifying residue is Lys-81 and/or Lys-217.

INTRODUCTION

Bovine heart mitochondrial L-malate dehydrogenase (MDH) (EC 1.1.1.37) is a dimeric enzyme (molecular mass 65 kDa) with identical subunits, which reduces oxaloacetate using NADH in preference to NADPH. The forward reaction is thermodynamically less favoured, the enzyme oxidizing L-malate and other 2-hydroxydicarboxylic acids in the presence of NAD⁺. Chemical modification of the NADH-binding site of pig heart MDH has been the subject of many investigations in the past [1–7]. However, for the bovine heart enzyme, there is little structural information about the active-site residues. It has been reported that bovine heart MDH has cysteine [8], histidine [8] and tyrosine [9] residues at the nucleotide coenzyme-binding site, as evidenced by chemical modification using only general protein reagents. Affinity labelling has not so far been applied in the case of bovine heart MDH to limit the extent of chemical modification to the region of the active site. Therefore MDH provides an appropriate system for investigating structure–function relationships within the active site, and possibly studying associated control sites on the enzyme by chemical means.

Anthraquinone dyes have been used in biochemical [10–23] and toxicological [24,25] studies, and in protein purification [26–29]. It has been recognized that aromatic sulphonated dyes, particularly those exhibiting an anthraquinone moiety, tend to bind preferentially to the active-site regions of globular proteins [11] and mimic the binding of naturally occurring anionic coenzymes such as NADH, ATP, CoA, flavins and folate [19]. We have previously described the use of the reactive dichlorotriazine anthraquinone dye Vilmax Blue A-R (VBAR) as a structural probe for labelling stoichiometrically the coenzyme-binding site of formate dehydrogenase [22]. The present study shows the affinity labelling of the coenzyme-binding site of bovine heart MDH, and identifies the modified residue responsible by employing both chemical and molecular-modelling techniques.

EXPERIMENTAL

Materials

NAD⁺ (crystallized lithium salt; approx. 100%), NADH (disodium salt; grade II; 98%), oxaloacetate (free acid) and crystalline BSA (fraction V) were obtained from Boehringer, Mannheim, Germany. HEPES and trypsin from bovine pancreas (grade III; 10 800 units/mg) were purchased from Sigma. VBAR was a gift from Dr. J. Mazza (Vilmax S.A., Buenos Aires, Argentina) and *N*^ε-fluoren-9-ylmethoxycarbonyl(Fmoc)-L-Lys-OH·CF₃COOH was a gift from Professor P. Kordopatis (Department of Pharmacy, University of Patras, Patras, Greece).

Bovine mitochondrial MDH was purified from acetone powder of bovine heart by chromatography on DEAE-agarose and CM-agarose essentially following the procedure of Thorne [30,31] and Anderton [32], and finally by affinity chromatography on Cibacron Blue 3GA-agarose adsorbent. The affinity-chromatography step was performed in 20 mM potassium phosphate buffer, pH 8.0, following by step elution with 0.5 M KCl in the same buffer. The preparation obtained exhibited a specific activity of 1300 units/mg (protein determination by the method of Lowry et al. [33]).

Assay of MDH

Enzyme assays were performed at 25 °C (10 mm pathlength) by a published method [34]. One unit of enzyme activity is defined as the amount that catalyses the conversion of 1 μ mol of oxaloacetate into L-malate/min.

Determination of protein concentration

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

Abbreviations used: MDH, malate dehydrogenase; VBAR, Vilmax Blue A-R; Fmoc, fluoren-9-ylmethoxycarbonyl; HPGFC, high-performance gel-filtration chromatography.

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MDH [8] or the Lowry method [33] with crystalline BSA (fraction V) as standard.

Enzyme-inactivation studies

Inactivation of MDH was performed in incubation mixture containing in 1 ml total volume (35 °C): Hepes/NaOH buffer, pH 8.5, 100 μ mol; VBAR, 0–50.1 nmol; enzyme, 1.3 units (MDH assay at 25 °C). The rate of inactivation was followed by periodically removing samples (20 μ l) for assay of enzyme 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.

Inactivation studies of MDH by VBAR in the presence of nucleotides (NAD⁺, NADH and ADP) and organic acid substrates (oxaloacetate and malate) were performed in a total volume of 1 ml (35 °C) and the reaction mixture contained: Hepes/NaOH buffer, pH 8.5, 100 μ mol; VBAR, 40 nmol; nucleotide or organic acid substrates, 1 μ mol; and MDH, 1.3 units.

In order to calculate the pK_a of the amino acid residue involved in MDH nucleophilic modification by VBAR, enzyme-inactivation experiments were performed at various pH values (7.03–8.57) in 100 μ mol of Hepes/NaOH buffer, containing 44 nmol of VBAR. A plot of 1/k_{obs.} versus [H⁺] gives a straight line from which the pK_a can be calculated (by unweighted linear-regression analysis) based on the equation [3,35]:

$$1/k_{\text{obs.}} = 1/k_{\text{int.}} + 1/k_{\text{int.}} \cdot ([\text{H}^+]/K_a)$$

where k_{obs.} is the observed rate of enzyme inactivation for a given concentration of dye, k_{int.} is the intrinsic pH-independent rate constant and K_a is the dissociation constant for the ionizing group.

Stoichiometry of NADH binding to MDH

The number of NADH-binding sites was determined by the method of Hummel and Dreyer [36] using high-performance gel-filtration chromatography (HPGFC) column [Protein PAK 300SW (Waters); 300 mm × 7.8 mm internal diameter; flow rate 0.5 ml/min]. Binding was measured at pH 6.5 in potassium phosphate buffer (0.1 M) over an NADH concentration range 1.0–20.0 μ M, employing 1.0 μ mol of MDH subunit. The NADH concentration was determined by its absorbance at 260 nm using a molar absorption coefficient of 14.3 litre·mmol⁻¹·cm⁻¹. Scatchard-plot analysis was performed using the Erithacus software program obtained from Sigma.

Stoichiometry of VBAR binding to MDH

MDH (10 nmol) in 100 mM Hepes/NaOH, pH 8.5, was inactivated with 35 nmol of VBAR at 35 °C until the remaining activity was less than 5%. The dye-inactivated enzyme was separated from the free dye by ultrafiltration (in an Amicon stirred-cell 8050 carrying a Diaflo YM10 ultrafiltration membrane; molecular-mass cut-off 10 kDa) after extensive washing with distilled water. Further separation was achieved by gel-filtration chromatography by applying the inactive dye–enzyme complex to a Sephadex G-25 column (9 cm × 1.6 cm) equilibrated with water, and collecting fractions (0.5 ml) at a flow rate of 10 ml/h. The solution with dye-inactivated MDH was then lyophilized and stored at –20 °C. The lyophilized VBAR–MDH covalent complex was diluted 5-fold with 8 M urea, and the absorbance was determined spectrophotometrically at 620 nm

using a molar absorption coefficient of 11.6 litre·mmol⁻¹·cm⁻¹ (8 M urea) [22]. The protein concentration was determined by the method of Lowry et al. [33]; no dye interference was observed in the protein determinations [21,37].

Tryptic digestion of the dye–enzyme (VBAR–MDH) covalent complex and peptide mapping using HPLC

Lyophilized VBAR–MDH covalent complex (100 μ g) was dissolved in 0.1 M NH₄HCO₃ solution, pH 8.3 (1 ml). The enzyme was digested by the addition of 2 μ g of trypsin. The digestion was allowed to continue overnight at 30 °C before the mixture was lyophilized and stored dry at –20 °C. Separation of the resulting peptides was achieved on a C₁₈ reverse-phase S5 ODS2 Spherisorb silica column (250 mm × 4.6 mm internal diameter). Analysis was effected with a water/acetonitrile linear gradient containing 0.1% trifluoroacetic acid (0–80% acetonitrile in 80 min) at a flow rate of 0.5 ml/min (absorbance at both 220 nm and 610 nm was measured).

Preparation of standard N^ε-VBAR–L-lysine (VBAR–Lys)

N^ε-Fmoc-L-Lys-OH·CF₃COOH (100 mg) was dissolved in dimethylformamide (5 ml) before being added to a solution (5 ml) of VBAR (60 mg; purity 61.3%, w/w). The pH was adjusted and kept throughout the reaction at 7.0–7.5. The reaction course was followed by analytical TLC with butan-1-ol/propan-2-ol/ethyl acetate/water (2:4:1:3, by vol.) as solvent. Spot visualization was effected first under visible light, and then after spraying with ninhydrin solution [0.1% (w/v) in butan-1-ol]. After approx. 3.5 h the reaction mixture was chromatographed on a lipophilic Sephadex LH-20 column, by the method of Labrou and Clonis [22]. After TLC analysis of collected fractions, the middle band eluted was identified as the desired product. Hydrolysis of the Fmoc group from N^ε-Fmoc-(N^ε-VBAR)–L-lysine was achieved by incubation of the product in a solution of piperidine (20%, v/v) for 1 h. After the hydrolysis step, the product was rechromatographed on the lipophilic Sephadex LH-20 column, and those fractions containing pure product were pooled, concentrated by 50% on a rotary evaporator under reduced pressure (at 50 °C), and the VBAR–Lys was lyophilized and stored desiccated at 4 °C. The purity of the final product was checked by TLC (positive reaction to ninhydrin) and HPLC in a water/acetonitrile linear gradient containing 0.1% trifluoroacetic acid (0–55% acetonitrile in 40 min) at a flow rate of 0.5 ml/min (A₆₁₀). TLC analysis was performed with butan-1-ol/propan-2-ol/ethyl acetate/water (2:4:1:3, by vol.) or butan-1-ol/acetic acid/water (4:1:1, by vol.) as solvent. The relative distance of the product with respect to VBAR as a reference was 0.9 and 0.88 respectively. HPLC analysis gave a retention time of 30.4 min.

Total acid hydrolysis of the VBAR–MDH covalent complex

VBAR–MDH covalent complex (100 μ g) was totally hydrolysed in 6.0 M HCl (1.0 ml) at 110 °C *in vacuo* for 24 h, and the HCl removed in a rotary evaporator under reduced pressure (55 °C). The conjugate N^ε-VBAR–L-Lys was treated under the same conditions of hydrolysis as above, in order to be used subsequently as a standard in TLC and HPLC analysis.

Identification of the reactive amino acid residue of MDH

Identification of the reactive amino acid residue of MDH was performed by HPLC and TLC analysis using as standard N^ε-

VBAR-L-Lys. HPLC analysis was performed on a C_{18} reverse-phase S5 ODS2 Spherisorb silica column (250 mm \times 4.6 mm internal diameter) with a water/acetonitrile linear gradient containing 0.1% trifluoroacetic acid (0–55% acetonitrile in 40 min) at a flow rate of 0.5 ml/min (A_{610}). TLC analysis was performed on precoated plastic sheets with silica gel 60 (0.2 mm; Merck) using as a solvent system butan-1-ol/propan-2-ol/ethyl acetate/water (2:4:1:3, by vol.) or butan-1-ol/acetic acid/water (4:1:1, by vol.).

Model generation

The protein model

The three-dimensional structures of MDH from porcine heart [38] and *Escherichia coli* [39] have been determined, and the *E. coli* co-ordinates are available through the Brookhaven database [40]. The two structures have 59% identity on the primary structure and a high degree of structural similarity. We have used the three-dimensional co-ordinates of the *E. coli* MDH structure to build a model of the binding-site region of pig mitochondrial MDH, since the structure of the latter is not yet available in the Brookhaven database. The model was based on the structural alignments of Gleason et al. [38], replacing in total 10 amino acid residues located within 0.5 nm (5 Å) of the NAD^+ cofactor and the organic acid substrate. These were in positions (*E. coli* numbering) 9 (A to S), 76 (S to P), 80 (A to P), 88 (S to D), 96 (G to T), 99 (K to A), 100 (N to T), 118 (T to S), 152 (I to V) and 215 (E to K). Of these, none was found to interact directly with the NAD^+ cofactor or the substrate, leading to the previously stated assumption [38] that the models of the substrate-binding sites for pig mitochondrial MDH and *E. coli* MDH are essentially identical.

The model of VBAR

This was built using structural information derived from X-ray structures available from the Cambridge Structural Database [41] for the anthraquinone, sulphonate and triazine groups. The model was constructed using the molecular graphics program Quanta-Charmm [42] on a Silicon Graphics Personal Iris graphics workstation and was superimposed on the NAD^+ cofactor of the *E. coli* MDH. The ligand position and conformation in the cofactor-binding site was based on structural information and optimization of ligand–enzyme interactions. After positioning of the anthraquinone part of the structure of VBAR, the conformation of the remaining structural parts was investigated to avoid poor contacts and to optimize hydrogen-bond formation and hydrophobic interactions. The final conformation was optimized by employing molecular graphics and energy-minimization techniques using the Charmm force field *in vacuo* [42]. During the energy minimization, the cores of the enzyme's amino acids were fixed at their crystallographic positions, whereas, on the dye, no geometrical restraints were applied.

RESULTS

The inactivation of MDH follows pseudo-first-order kinetics (Figure 1) in the presence of the dichlorotriazine VBAR (pH 8.5; 35 °C). A control sample of MDH, in the absence of VBAR, displays no loss of activity during the same period. Furthermore the activity of VBAR-inactivated MDH cannot be regained on incubation with NADH, NAD^+ or ADP (10 mM), or by extensive dialysis, gel filtration on Sephadex G-25 or ultrafiltration. Linearity in semilogarithmic plots of the irreversible inactivation process is observed with VBAR concentrations 10.2–50.1 μ M.

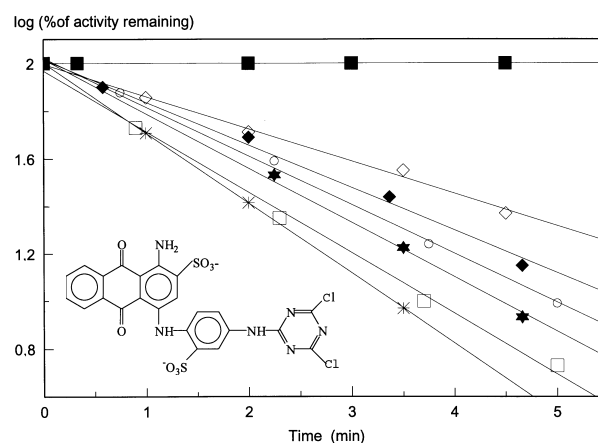


Figure 1 Time course for the inactivation of MDH by VBAR at pH 8.5 and 35 °C

■, No VBAR; ◇, 10.2 μ M; ◆, 14.5 μ M; ○, 17.4 μ M; ★, 21.7 μ M; □, 29.0 μ M; ✱, 50.1 μ M. Inset: The structure of VBAR.

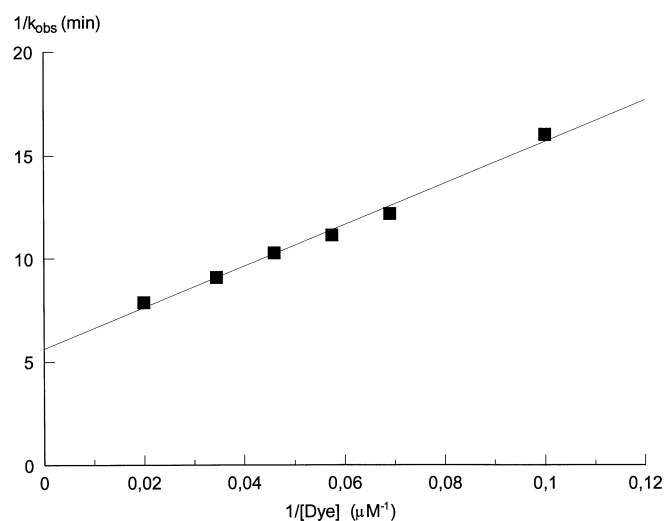


Figure 2 Effect of VBAR concentration on the observed rate of inactivation (k_{obs}) of MDH by VBAR expressed as a double-reciprocal plot

VBAR concentration ranged from 10.2 to 50.1 μ M. The slope and intercept of the secondary double-reciprocal plot were calculated by unweighted linear-regression analysis.

The following mathematical equation describes the reaction between an active-site-directed reagent (e.g. reactive dye D) and an enzyme (E):

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

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 [20,22,23,42,43]. Regarding MDH inactivation, a plot of $1/k_{obs}$ versus $1/[D]$ for several concentrations of VBAR yields a straight line with a positive ordinate intercept (k_3) of 0.16 min^{-1} and a slope corresponding to an apparent dissociation constant of 14.4 μ M (Figure 2). These results are consistent with the conclusion that the dye forms a Michaelis–

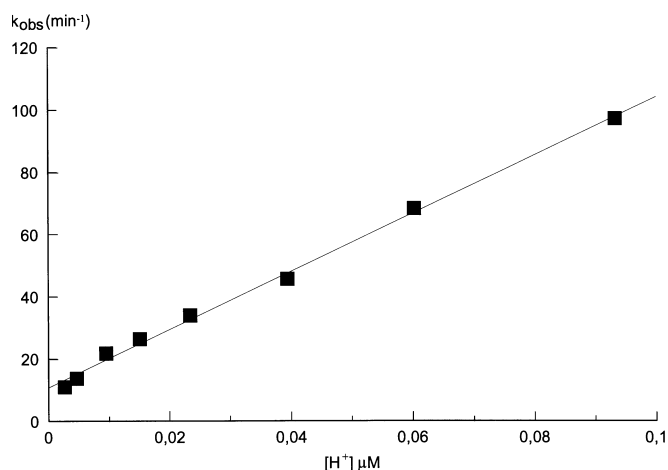


Figure 3 pH-dependence of MDH inactivation by VBAR

The variation of the reciprocal of the observed rate of inactivation with proton concentration is shown. The reaction mixture contained 44 μM VBAR and 100 mM Hepes/NaOH buffer, pH 7.03–8.57.

type reversible complex E–D, and the formation of the covalent product is rate-limiting [43–45].

The effect of pH on enzyme inactivation was measured to obtain more information about the mechanism of the inactivation. The rate of inactivation increased as the pH was raised from 7.03 to 8.57, indicating that the reaction strongly depends on the nucleophilicity of a deprotonated group. A study of the effect of pH on enzyme inactivation allows the calculation of the pK_a of the nucleophile involved in the inactivation reaction. A plot of $1/k_{obs}$ versus $[H^+]$ should be linear on condition that the inactivation reaction depends only on the ionization of a single residue [3,35]. According to Figure 3, linearity occurs when the variation of the reciprocal of the apparent rate of MDH inactivation by VBAR is plotted against hydrogen ion concentration, leading to a pK_a of 8.05 ± 0.13 .

The binding of NADH to bovine heart MDH was studied by the gel-filtration technique of Hummel and Dreyer [36]. The data obtained by the HPGFC procedure were analysed by Scatchard plot [46]. According to Figure 4, in 0.1 M phosphate buffer, pH 6.5, $n = 1.9 \pm 0.05$ was obtained, indicating the presence of two NADH-binding sites per MDH dimer. The dissociation constant calculated from the plot was 5.6 μM, a value that is in good agreement with those reported previously [3,7].

To determine the stoichiometry of VBAR binding, MDH was completely inactivated by the dye, and the dye–enzyme covalent complex was resolved from free dye by gel filtration on Sephadex G-25 and ultrafiltration. The molar ratio [VBAR]/[MDH active site] was determined by measuring the dye spectrophotometrically in urea solution, and the protein by the method of Lowry et al. [33]. The molar ratio of VBAR to MDH active site was 1:1, using a subunit molecular mass 33 kDa [8], indicating a specific interaction between dye and protein. Furthermore VBAR-modified MDH was digested with trypsin and the resulting peptides were mapped by reverse-phase HPLC on a C₁₈ silica column (Figure 5). Essentially, a single VBAR-labelled peptide emerged from the column, indicating that VBAR attacks the one and only peptide per MDH subunit.

When NAD⁺, NADH or ADP was included in the inactivation reaction mixture, the rate of MDH inactivation by VBAR decelerated to various degrees. As shown in Table 1, at a

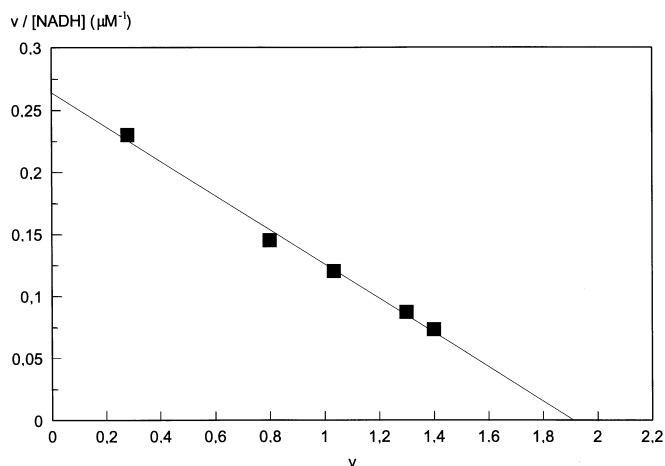
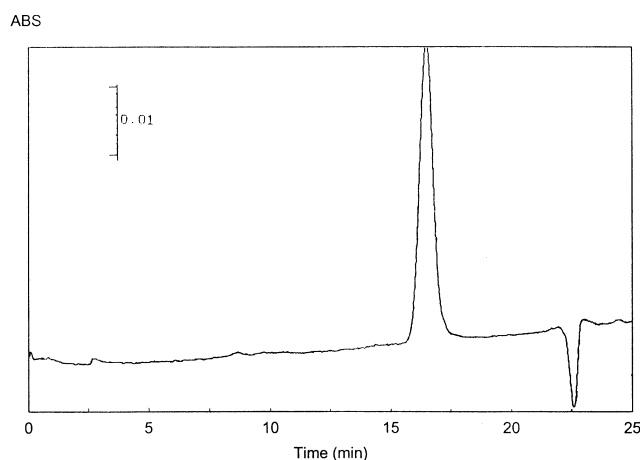


Figure 4 Binding of NADH to MDH

Top, binding of NADH to MDH obtained by HPGFC in 100 mM potassium phosphate buffer, pH 6.5. Column effluents were monitored by their A_{260} (ABS) (scale in inset is arbitrary units). HPGFC runs were performed as described in the Experimental section. The trough at 22.7 min, corresponding to the depletion of NADH from the solution by binding to MDH, was used for the calculation of the amount of bound NADH ($NADH_b$) using as a standard the area of 11 μmol of NADH. Bottom, binding data plotted according to Scatchard, where $v = \mu\text{mol}$ of $NADH_b/\mu\text{mol}$ of total MDH, and $[NADH]$ is the concentration of free nucleotide under equilibrium in the column.

concentration of 1 mM the specific nucleotides were found to protect MDH partially against inactivation, with NADH giving the best protection, which is in agreement with their relative affinity constants [3]. Although oxaloacetate and malic acid alone show no protection of MDH inactivation against VBAR, mixtures of oxaloacetate and NAD⁺ and malic acid and NADH enhanced the protection, demonstrating the formation of ternary complexes exhibiting an improved protective effect.

To identify the MDH residue modified by VBAR, we originally employed chemical methods. The VBAR–MDH covalent complex was acid-hydrolysed totally before being subjected to reverse-phase HPLC and TLC analysis. The results from a typical HPLC run (Figure 6) indicate that a single blue compound emerged with the same retention time (t_R 30.4 min) as that for the *N*^ε-VBAR–L-Lys standard. The retention times for free dyes, under the same chromatography conditions, were 38.2 min for VBAR and 48.5 min for Cibacron Blue 3GA. Furthermore TLC analysis revealed the presence of a single blue compound

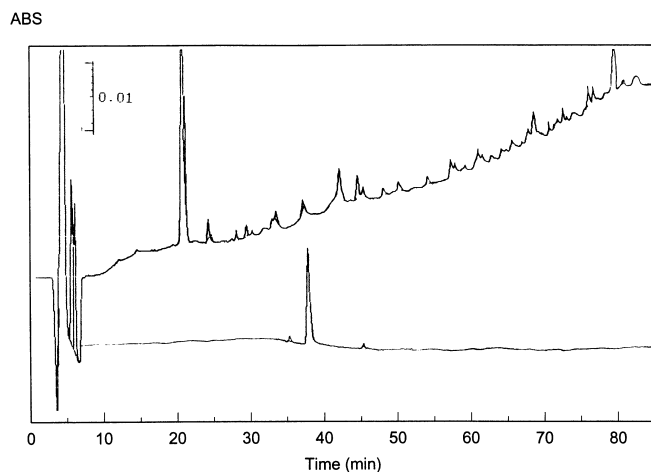


Figure 5 Reverse-phase HPLC of the tryptic digest of MDH-VBAR covalent complex

The tryptic digest of the enzyme was injected on to a C_{18} reverse-phase S5 ODS2 Spherisorb silica column (250 mm \times 4.6 mm internal diameter) (flow rate 0.5 ml/min). Labelling and digestion of the enzyme and the HPLC were performed as described in the Experimental section. Eluted peptides were monitored at both 220 nm (upper curve) and 610 nm (lower curve); scale in inset is arbitrary units. Abbreviation: ABS, absorbance.

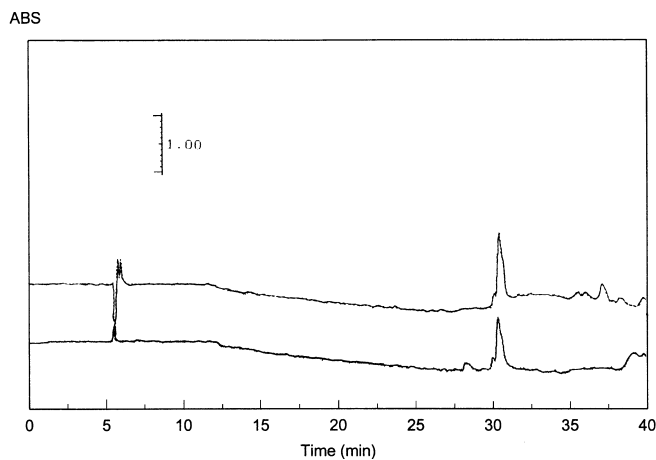


Figure 6 Reverse-phase HPLC of the total acid hydrolysate of MDH-VBAR covalent complex (lower curve) and the standard N^{ϵ} -VBAR-L-Lys (upper curve)

Hydrolysed VBAR-enzyme complex and the standard N^{ϵ} -VBAR-L-Lys were injected on to a C_{18} reverse-phase S5 ODS2 Spherisorb silica column (250 mm \times 4.6 mm internal diameter) (flow rate 0.5 ml/min). Labelling, acid hydrolysis of the enzyme and HPLC were performed as described in the Experimental section. Eluted compounds were monitored at 610 nm; scale in inset is arbitrary units. Abbreviation: ABS, absorbance.

Table 1 Effect of competing nucleotides and organic acid substrates on the protection of MDH from inactivation by VBAR (40 μ M) at pH 8.5 and 35 $^{\circ}$ C

All the compounds were tested at a concentration of 1 mM. Protection is given as the percentage reduction in the observed rate of inactivation.

Competing compounds	Protection (%)
VBAR	—
NADH	40.1
NAD ⁺	25.4
ADP	17.0
NADH plus L-malic acid	57.5
NAD ⁺ plus oxaloacetic acid	36.2

indistinguishable from the N^{ϵ} -VBAR-L-Lys standard, using two solvent systems, one for amino acid analysis (butan-1-ol/acetic acid/water; 4:1:1, by vol.) and one for dye analysis (butan-1-ol/propan-2-ol/ethyl acetate/water; 2:4:1:3, by vol.) [22].

The above results were in agreement with those obtained from molecular-modelling studies. From the protein model it is evident that the only nucleophiles within or close to the binding site, and accessible to the bound VBAR for covalent modification, are the ϵ -amino groups of Lys-81 (Figure 7, top) and Lys-217 (Figure 7, bottom), of which the former is located at the entrance of the binding site. Favourable conditions for initial strong binding of VBAR are created by (i) hydrophobic interactions between the anthraquinone moiety and hydrophobic residues (Leu, Val and Tyr) of the nucleotide-binding area, (ii) hydrogen-bond formation between the amino group of the anthraquinone moiety and Asp-34, and (iii) exposure of the polar sulphonate and carbonyl groups, leading to favourable entropic contribution to the binding energy.

DISCUSSION

Several nucleotide-dependent enzymes are susceptible to chemical modification by dichlorotriazine dyes [20,22,37] and to a lesser extent by monochlorotriazine dyes, e.g. CB3GA [44] and Procion green H-4G [21]. Time- and dye-concentration-dependent inactivation of MDH by VBAR is evident. The pseudo-first-order kinetics obtained for MDH inactivation indicates that the phenomenon occurs through the initial formation of a reversible Michaelis binary complex followed by subsequent formation of a covalent complex.

The ability of specific ligands (e.g. substrates and inhibitors) to prevent enzyme inactivation by an irreversible inhibitor (label) is usually taken as evidence that the inhibitor is either active-site-directed or affected by conformation changes resulting from ligand binding at the active site [45]. The relatively high protection afforded by NADH against MDH inactivation by reactive VBAR resembles the profile obtained when the active-site-directed reagents iodoacetamide and *N*-ethylmaleimide are used [8]. Furthermore two ternary complexes, MDH-NAD⁺-oxaloacetate and MDH-NADH-malic acid, could be formed. No evidence was obtained for binary complex-formation (MDH-oxaloacetate and MDH-malic acid). This finding is consistent with a compulsory-order mechanism [48].

Gregory [8], working with bovine heart MDH, suggested the presence of a single NADH-binding site per dimer, thus one active site per MDH molecule. This is in direct contrast with the results obtained in the present study according to which two NADH-binding sites per dimer are present (Figure 4). This finding is strengthened further by affinity-labelling experiments with MDH. The specificity of the VBAR interaction with MDH is demonstrated by both the stoichiometry of incorporation, indicating labelling of one residue per active site, and peptide mapping after tryptic digestion, which revealed essentially one VBAR-labelled peptide (Figure 5). The above findings can be taken as strong evidence that VBAR binds irreversibly to the nucleotide-binding domain of the MDH subunit and in equimolar

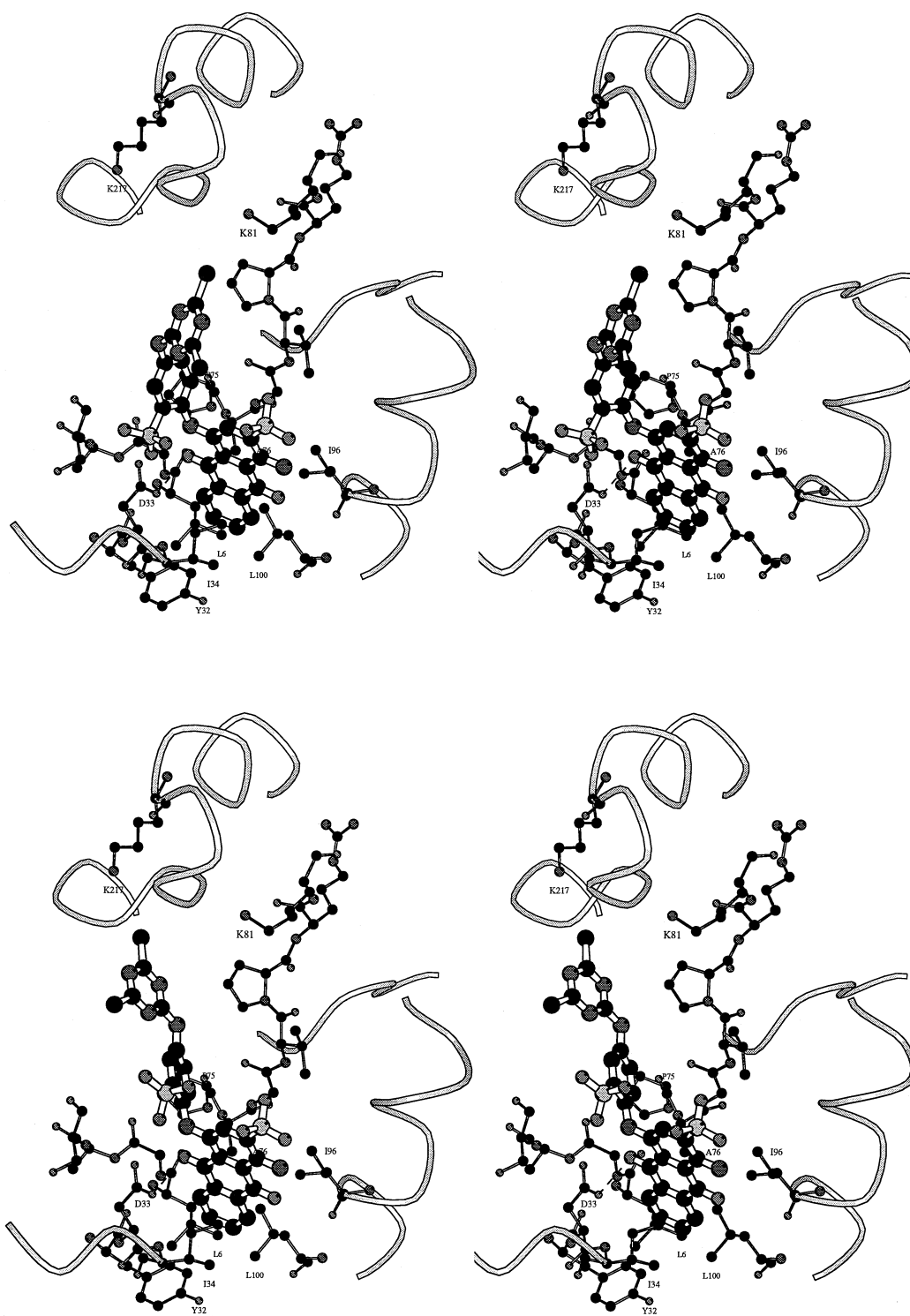


Figure 7 Stereodiagrams of VBAR in the coenzyme-binding site of pig mitochondrial MDH

Top, in a conformation close to Lys-81; bottom, in a conformation close to Lys-217. The diagrams were produced using the program MOLSCRIPT [47].

ratio. Consequently, one could assume that each subunit has the same peptide bearing the reactive residue, responsible for the nucleophilic displacement of chlorine from the triazine ring of VBAR. Furthermore total acid hydrolysis of the VBAR-labelled MDH, followed by TLC and HPLC analysis using N^6 -VBAR-L-

Lys as standard, has shown that the modifying reactive residue is a lysine (Figure 6). The pK_a value of this residue was found to be 8.05 ± 0.13 , which is in good agreement with the essential lysine residue of glutamate dehydrogenase [49], but is considerably lower than the value expected for the free amino acid.

The low pK_a presumably reflects the requirement for a deprotonated reactive residue below the pH necessary for maximum MDH activity.

Both from the chemical studies and the molecular-modelling approach described here, it became evident that the only nucleophiles accessible to VBAR for covalent modification of MDH are the ϵ -amino groups of Lys-81 and Lys-217, of which the former is located at the entrance of the binding site. The binding pattern observed between VBAR and MDH is similar to that reported for the surface-located Lys-58 in the inactivation of pig heart L-lactate dehydrogenase [20], but different from that for Cys-174 in the inactivation of horse liver alcohol dehydrogenase, a residue that co-ordinates with the catalytically important zinc atom [37]. Although the actual role of the lysine residue is not fully understood, it is likely that the same residue is also present in pig heart MDH, which can be labelled by 5'-[*p*-(fluoro-sulphonyl)benzoyl]adenosine [7] and pyridoxal 5'-phosphate [4]. It is conceivable that the conservation of this amino acid reflects a uniform mechanism in coenzyme binding in the two enzymes. Furthermore, in view of the similar susceptibility of a series of NAD(P)⁺-dependent dehydrogenases to reversible inactivation by pyridoxal 5'-phosphate, it seems possible that an essential lysine residue might be a common feature of their structure [5]. This lysine was considered to be essential to the catalytic activity. However, this suggestion is probably not applicable, at least for MDH, where the modifying lysine residues are remote from the active site. Their modification locks the enzyme binding site and results in steric hindrance of the catalytic reaction.

The high absorption coefficient, stability, solubility and reactivity of the anthraquinone dichlorotriazine dye VBAR, together with its affinity for nucleotide-binding sites, make it a useful covalent label. Furthermore it is believed that, when this property is combined with a suitable substrate-mimetic element, it may lead to new chimaeric biomimetic affinity ligands, able to recognize the coenzyme-binding site (via the VBAR moiety) and the catalytic site (via the substrate-mimetic moiety) of target enzymes.

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