Effect of selective thiol-group derivatization on enzyme kinetics of (*R*)-3-hydroxybutyrate dehydrogenase

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(*R*)-3-Hydroxybutyrate dehydrogenase (BDH) is a phosphatidylcholine-requiring tetrameric enzyme with two thiol groups (SH-1 and SH-2) per protomer. By first protecting the more rapidly reacting thiol group (SH-1) with diamide [1,1'-azobis-(*NN'*-dimethylformamide), DM] to form DM(SH-1)BDH, SH-2 can be selectively derivatized by reaction with maleimide reagents such as 4-maleimido-2,2,6,6-tetramethylpiperidine-*N*-oxyl (MSL), which gives DM(SH-1)MSL(SH-2)-BDH. Reduction with dithiothreitol (DTT) regenerates SH-1, yielding MAL(SH-2)BDH (where MAL is the diamagnetic reduction product of MSL-BDH and DTT). The enzymic activity of DM(SH-1)BDH is decreased to approx. 4% relative to the native purified enzyme, and the apparent K_m for substrate, K_m^{BOH} , is increased approx. 100-fold. Reduction of DM(SH-1)BDH with DTT regenerates SH-1 and restores normal enzymic function. Modification of SH-2 with piperidinylmaleimide

[MAL(SH-2)BDH] diminishes enzymic activity to approx. 35% of its original value, but has no significant effect on apparent K_m^{BOH} . The doubly derivatized enzyme, DM(SH-1)MSL(SH-2)-BDH, has lower enzymic activity [about half that for DM(SH-2)-BDH] and a yet higher apparent K_m^{BOH} than DM(SH-1)BDH. Derivatization of SH-2 with different maleimide reagents results in diminished activity approximately proportional to the size of the maleimide substituent, suggesting that this inhibition is steric. Whereas modification of SH-1 results in marked changes in kinetic parameters (increased apparent K_m and reduced apparent V_{max}), derivatization of SH-2 has a lesser effect on enzymic function. Thus SH-1 is postulated to be closer to the active centre than is SH-2, although neither is involved in catalysis, since: (1) the activity of the derivatized enzyme is not abolished; and (2) activity can be enhanced by increasing substrate (and cofactor) concentrations.

INTRODUCTION

(*R*)-3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) (BDH) has a specific requirement for phosphatidylcholine (PC) for enzymic activity [1] and is one of the best-studied examples of a lipid-requiring enzyme [2–5]. BDH was first highly purified from bovine heart mitochondria by Bock and Fleischer [6]. The purified apoenzyme is devoid of lipid and inactive, but the activity can be restored by reconstitution with phospholipid vesicles containing PC [7,8]. Activation is specific for the phosphocholine moiety of PC [9] and appears to involve an allosteric mechanism [10] in which PC enhances binding of nucleotide by more than an order of magnitude [11,12]. BDH consists of a single type of subunit (M_r approx. 31 500) [6] and, by target inactivation analysis, is tetrameric both in the mitochondrial membrane and when reconstituted with phospholipid vesicles [13]. Native BDH in the mitochondrial membrane and the purified enzyme reconstituted with the mixture of mitochondrial phospholipids (MPL) has the same ordered sequential reaction mechanism (Ordered Bi Bi) [14] and in this regard is similar to the kinetic mechanism for a number of other dehydrogenases [15,16].

Each protomer of the BDH homotetramer contains six cysteine residues, two of which are reactive to thiol-group-reactive

reagents and the other four exist as disulphide bridges [17,18]. The two free thiol groups, SH-1 and SH-2, are distinguished on the basis of their reactivity with maleimide reagents or 'diamide' [18]. Chemical modification of the more reactive cysteine, designated SH-1, markedly diminishes enzyme activity [17,18]. In the presence of NAD^+ or $NADH$, only two SH-1 residues per tetramer are susceptible to derivatization [19], consistent with SH-1 being in the vicinity of the nucleotide-binding site, as confirmed by its reaction with arylazido- NAD^+ [20]. The primary sequences of rat liver [21] and human heart [22] BDH have recently been obtained from cDNA cloning studies. Analysis of the predicted amino acid sequence of human heart BDH (297 residues, M_r 33 117) indicates that SH-1 is Cys-242 and located in the domain (C-terminal third of the sequence) that likely contains elements which determine the substrate specificity for the enzyme and the phospholipid-binding site(s) [22].

The selective reaction of diamide with SH-1 and BDH and its facile removal with dithiothreitol (DTT) provides an approach to obtain selective derivatization of the second thiol group (SH-2) with maleimide reagents [18]. This approach has been used in these studies to selectively derivatize BDH at SH-1 and/or SH-2 to determine effects on enzymic function, including catalytic rate and apparent Michaelis constants for coenzyme and substrate.

Abbreviations used: BDH, (*R*)-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30); BDH:MPL, BDH (tetrameric) reconstituted with mitochondrial phospholipid vesicles; BOH, 3-hydroxybutyrate [(*R,S*) racemic mixture or (*R*)-isomer, the substrate for BDH, as indicated]; CPM, 7-diethylamino-3-(4'-maleimidophenyl)-4-methylcoumarin; DM, 'diamide' [1,1'-azobis-(*NN'*-dimethylformamide)]; FLM, fluorescein-5-maleimide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; MAL, diamagnetic reduction product of 4-maleimido-2,2,6,6-tetramethylpiperidine *N*-oxyl (MSL) and dithiothreitol (DTT); MPL, the mixture of mitochondrial phospholipids; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine.

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MATERIALS AND METHODS

Reagents and general methodology

The general methodology and analytical procedures were essentially as described previously [8,10,12,18], except as noted below. The thiol-group-derivatizing reagents, 1,1'-azobis-(*NN'*-dimethylformamide) (diamide, DM), *N*-ethylmaleimide (NEM), and 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxyl (MSL) were obtained from Sigma Chemical Co. 7-Diethylamino-3-(4'-maleimidophenyl)-4-methylcoumarin (CPM) and fluorescein-5-maleimide (FLM) were from Molecular Probes (Eugene, OR, U.S.A.).

Dialysis membrane tubing (Spectrapor Type 2; M_r cut-off 12000–14000; 10 or 25 mm flat width; Spectrum Medical Industries, Los Angeles, CA, U.S.A.) was treated as described by Brewer et al. [23], with modifications. The tubing was soaked for 1 h in 1% acetic acid, rinsed with deionized water, and then heated to 60 °C sequentially three times in 1% Na_2CO_3 containing 1 mM EDTA. Finally, the tubing was rinsed with deionized water at room temperature, heated to 60 °C in water, rinsed, and stored at 4 °C in deionized water.

Purification of BDH and reconstitution with phospholipid vesicles

Purified BDH, devoid of lipid (apo-BDH), was isolated from bovine heart mitochondria as described in [25] and stored at –196 °C in 0.4 M LiBr/5 mM Hepes/NaOH, pH 7.0, containing 5 mM dithiothreitol. Vesicles of mitochondrial phospholipids (MPL) were prepared as described previously [8,10,24]. The BDH:MPL complex was prepared at 0.1 mg/ml in 20 mM Tris/HCl (pH 8.1)/1 mM EDTA/10 mM DTT/5 mM NAD^+ with MPL vesicles (100 μg of phosphorus/mg of BDH) as described in [8]. The initial specific activities ranged from 80 to 130 μmol of NAD^+ reduced $\cdot\text{min}^{-1}\cdot\text{mg}$ BDH $^{-1}$ for different preparations of BDH. After dialysis versus three 100 vol. of dialysis buffer (50 mM NaCl/5 mM Hepes/1 mM EDTA, pH 7.0 at 4 °C) to remove DTT and NAD^+ , the BDH:MPL complex was allowed to react with thiol-group-derivatizing agents.

Selective derivatization of the thiol groups of BDH:MPL

Two derivatization pathways are illustrated in Scheme 1. Pathway 1 is the single-step modification of the more reactive cysteine (SH-1) of BDH:MPL (BDH-I) with maleimide spin label (MSL), whereas Pathway 2 consists of three sequential steps yielding BDH-II, BDH-III and BDH-IV as follows: (1) derivatization of SH-1 with DM (BDH-II) (this step can be reversed by reduction with DTT [18]); (2) reaction of BDH-II with MSL to form the doubly derivatized DM(SH-1)MSL(SH-2)BDH:MPL (BDH-III); and (3) reduction of BDH-III with DTT to yield the diamagnetic MAL(SH-2)BDH:MPL (BDH-IV).

Covalent derivatization at SH-1 with MSL (Pathway 1) was carried out under conditions similar to those described [17] for derivatization with NEM. MSL (10 mM in anhydrous ethanol) was added to dialysed BDH:MPL (0.1 mg of BDH $\cdot\text{ml}^{-1}$; approx. 3 μM BDH protomer) in MSL/BDH molar ratios of 5:1 and 30:1 (i.e., 15 or 90 μM MSL), both of which resulted in essentially complete derivatization of SH-1 with MSL (results not shown) as previously reported with NEM [17,18]. For labelling SH-2 (Pathway 2), the more reactive SH-1 was protected by prior derivatization with DM, essentially as described by Fleer et al. [18]. DM (100 mM in ethanol) was added (final concn. 1 mM) to dialysed BDH:MPL (0.1 mg/ml in argon-

Scheme 1 Derivatization of BDH:MPL with thiol reagents

Two pathways for alkylation of BDH:MPL (BDH-I) with maleimide spin label (MSL) at SH-1 or SH-2 are illustrated. In Pathway 1, the reaction with 5 equiv. of MSL per protomer results in essentially complete (and irreversible) inactivation of the enzyme. In Pathway 2, the more reactive SH-1 is protected by reaction with 'diamide' (DM) to obtain DM(SH-1)BDH:MPL (BDH-II). The thiol can be regenerated by reducing BDH-II with DTT [18]. Reaction of BDH-II with 50 equiv. of MSL yields DM(SH-1)MSL(SH-2)BDH:MPL (BDH-III). Reduction of BDH-III with 50 mM DTT yields the product MAL(SH-2)BDH:MPL (BDH-IV), in which the free thiol (SH-1) is regenerated and the nitroxide of MSL is reduced.

saturated 50 mM NaCl/1 mM EDTA/5 mM Hepes, pH 7.0) and the reaction mixture stored on ice under an argon atmosphere for 45 min to ensure complete reaction of SH-1. In general, the reaction of SH-1 with DM was complete within 30 min, as monitored by the loss of enzymic activity. The reaction mixture, DM(SH-1)BDH (BDH-II), was dialysed 1 or 2 times against 100 vol. of dialysis buffer to remove excess DM, and then MSL (from a 10 mM stock solution in dry ethanol) was added to a final ratio of 50 mol of MSL per mol of BDH monomer (150 μM MSL; 3 μM BDH). After 6 h reaction under argon, excess MSL was removed by dialysis against 100 vol. of dialysis buffer, yielding DM(SH-1)MSL(SH-2)BDH (BDH-III). The final product, MAL(SH-2)BDH:MPL (BDH-IV), was obtained by reduction of BDH-III with DTT (typically 50 mM) at 5 °C, resulting in partial re-activation of the enzyme, with BDH activity reaching a plateau approx. 2 h after adding the reducing agent.

Figure 1 Activities of BDH:MPL and its sequential Pathway 2 derivatives under five assay conditions

The enzymic activities of BDH:MPL (BDH-I) and its Pathway 2 derivatives (BDH-II, -III and -IV; see Scheme 1) are shown in the four panels. For each panel, activities were measured in the assay medium (without DTT) described in the Materials and methods section, but with five assay conditions (A–E) in which the amounts of NAD⁺ and (*R*)-BOH were each varied by a factor of 10. The total [Na⁺] was either 40 mM for A (standard assay condition) or 240 mM for B–E (high salt). The concentration (in mM) of substrate, cofactor and total Na⁺ in each of the assay mixtures A–E is given below the key to the bar symbols at the bottom of the Figure. Activities are expressed relative to the activity of unlabelled BDH:MPL measured in condition A, which ranged between 79 and 122 μmol of NAD⁺ reduced/min per mg of BDH for different preparations. Data shown are from a typical labelling experiment.

Enzyme activity and kinetic parameters

The BDH activity was measured spectrophotometrically essentially as described previously, measuring the reduction of NAD⁺ to NADH at 340 nm [6,8]. The standard assay medium (37 °C) consisted of potassium phosphate (10 mM; pH 7.35), EDTA (0.5 mM), BSA (0.04%), ethanol (1.27%), DTT (2 mM), disodium NAD⁺ (5 mM), and sodium (*R,S*)-BOH (3-hydroxybutyrate) (20 mM). For DM derivatives of BDH, DTT was omitted from the enzyme assay. For comparison with previous studies [8,17,26], two fixed concentrations of Na⁺, 40 mM or 240 mM, were used with five variants (A–E) of the standard medium (see Figure 1). The enzymic reaction was initiated by addition of substrate and a computer program BDHACTV in HP-BASIC language was used for data acquisition and calculations (see Dalton [27]).

To determine the apparent K_m for substrate [*(R)*-BOH] and nucleotide (NAD⁺) and apparent V_{max} values, enzymic activity was measured as a function of either substrate or NAD⁺ concentration in the assay medium described above but with either 40 mM or 240 mM total Na⁺ as indicated. For BDH:MPL at 40 mM Na⁺, [*(R,S)*-BOH] ranged from 0.5 to 20 mM (at 5 mM NAD⁺) and [NAD⁺] ranged from 0.05 to 5 mM [at 20 mM (*R,S*)-BOH]. Derivatized forms of BDH:MPL were studied with [*(R,S)*-BOH] ranging from 1 to 200 mM (at 5 or 20 mM NAD⁺) and [NAD⁺] from 0.1 to 20 mM [at 200 mM (*R,S*)-BOH]. From these data, apparent V_{max} and K_m were calculated by non-linear regression analysis of specific-activity-versus-concentration data using the equation $Y = (A \cdot X)/(B + X)$ (rectangular hyperbolic function) with bi-square weighting [28] to reduce the influence of outlying points upon the calculated best fit.

RESULTS AND DISCUSSION

Selective site derivatization of SH-1 and SH-2

The activities of BDH:MPL (BDH-I) and three selectively modified thiol derivatives of BDH, DM(SH-1)BDH:MPL (BDH-II), DM(SH-1)MSL(SH-2)BDH:MPL (BDH-III), and MAL(SH-2)BDH:MPL (BDH-IV) (Scheme 1) are compared (Figure 1) using five different assay conditions. Condition A (the left bar in each panel) is comparable with those used previously [8,14]. In addition, BDH activity was measured at high total [Na⁺] (assays B–E) using two concentrations of substrate and/or cofactor (each varied 10-fold). Modification of SH-1 by DM results in a 50-fold decrease in enzymic activity measured using the standard assay (condition A, shown by solid bars, ■) (BDH-II versus BDH-I). Alkylation of SH-2 results in a 2.5-fold decrease in activity (condition A) for BDH-IV versus BDH-I and BDH-III versus BDH-II (Figure 1).

High [Na⁺] is inhibitory (assay condition C versus A) for SH-1 free forms of the enzyme (BDH-I and -IV), but less so for the SH-1-modified forms of the enzyme (BDH-II and -III) (Figure 1, left and right panels respectively). For BDH-II and -III (right-hand-side panels in Figure 1), a 10-fold increase in substrate from 10 to 100 mM (conditions C and E) resulted in about a 4-fold enhancement in activity, while for the SH-1-free forms (BDH-I and -IV, left-hand-side panels), only a modest (~20%) increase in activity is obtained at high substrate. In all four forms of the enzyme, a 10-fold increase in [NAD⁺] (assay conditions C versus B and E versus D) resulted in an average 2-fold increase in activity (Figure 1). These results indicate an increase in K_m^{BOH} for BDH-II and BDH-III, with little effect on $K_m^{\text{NAD}^+}$ and are consistent with measured kinetic parameters for the thiol derivatives (see below).

Derivatization of BDH:MPL with MSL at SH-1 yields MSL(SH-1)BDH:MPL (pathway 1, Scheme 1), with relative activities in assay conditions A to E (results not shown) comparable with those for DM(SH-1)BDH:MPL (BDH-II). In this regard, previous studies with BDH derivatized at SH-1 with 1 equiv. of cyanide or Ellman's reagent exhibited a similar enhancement in activity (~3-fold) by high substrate [26] as found in the studies reported here for the DM derivative (BDH-II). Thus increased activity at high substrate is characteristic of SH-1-modified BDH. The enzymic activity studies for the different derivatized forms of BDH-MPL yield a profile (see Figure 1) that is diagnostic for the enzyme modified at SH-1, i.e. the activity of SH-1-modified BDH is enhanced (~4-fold) by high substrate. For the DM derivative of BDH (BDH-II), SH-1 and enzymic activity (>90%) were restored by reduction with DTT, as reported previously [18]. In addition, after treatment of BDH-II with DTT, relative activities in the profile assay series A–E

Table 1 Enzyme activity and kinetic parameters for BDH:MPL and its thiol derivatives

BDH:MPL (BDH-I) and the alkylated derivatives (Pathway 2) (BDH-II, -III and -IV) were prepared as described in Scheme 1. BDH activities, measured in the absence of DTT, are given for assay conditions A and E (see Figure 1). Activities and apparent $V_{\max}$ values are expressed as a percentage of control (dialysed BDH:MPL in assay condition A) which varied between 79 and 130 μmol of NAD^+ reduced $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ for different preparations. Control samples (stored at 4 °C for 48 h) retained approx. 90% of original activity. Kinetic parameters [apparent $V_{\max}$ and K_m values for (*R*)-BOH or NAD^+] were obtained either at 40 mM or at 240 mM Na^+ as described in the Materials and methods section. Results are means $\pm$ S.E.M. for the number of determinations given in parentheses.

Enzymic function	BDH:MPL (BDH-I)	DM(SH-1)BDH:MPL (BDH-II)	DM(SH-1)MSL(SH-2)BDH:MPL (BDH-III)	MAL(SH-2)BDH:MPL (BDH-IV)
(I) Enzymic activity (% of control)				
(1) 10 mM (<i>R</i>)-BOH (condition A)	100	4.0 $\pm$ 1.0 (5)	1.9 $\pm$ 0.4 (3)	31 $\pm$ 3 (3)
(2) 100 mM (<i>R</i>)-BOH (condition E)	59 $\pm$ 2 (4)	18 $\pm$ 3 (5)	5.9 $\pm$ 1.3 (4)	23 $\pm$ 5 (4)
(II) Apparent $V_{\max}^{\text{BOH}}$ (% of control)				
(1) [Na^+] = 40 mM	119 $\pm$ 5 (2)			
(2) [Na^+] = 240 mM	64 $\pm$ 4 (5)	32 $\pm$ 11 (2)	25 (1)	23 $\pm$ 1 (2)
(III) Apparent $V_{\max}^{\text{NAD}^+}$ (% of control)				
(1) [Na^+] = 40 mM	116 $\pm$ 10 (3)			
(2) [Na^+] = 240 mM†	60 $\pm$ 1 (4)	21 $\pm$ 1 (2)	7.3 $\pm$ 0.2 (2)	24 (1)
(IV) Apparent K_m^{BOH} (mM)				
(1) Total [Na^+] = 40 mM	1.2 $\pm$ 0.4 (2)			
(2) Total [Na^+] = 240 mM	2.2 $\pm$ 0.4 (5)	200 $\pm$ 45 (2)	290 (1)	1.7 $\pm$ 0.3 (2)
(V) Apparent $K_m^{\text{NAD}^+}$ (mM)				
(1) [Na^+] = 40 mM	0.32 $\pm$ 0.01 (3)			
(2) [Na^+] = 240 mM	0.27 $\pm$ 0.01 (4)	1.3 $\pm$ 0.3 (2)	1.9 $\pm$ 0.2 (2)	0.47 (1)

* Values for apparent $V_{\max}$ ($V_{\max}^{\text{BOH}}$) and corresponding apparent K_m for (*R*)-BOH (K_m^{BOH}) given in parts II and IV respectively of this Table are from data similar to those shown in Figure 2. With BDH-I, (*R*)-BOH, added as the racemic mixture, was varied up to 10 mM (results not shown). The [NAD^+] was 5 mM for BDH-I and BDH-IV (free SH-1 forms) or 20 mM for BDH-II and BDH-III (alkylated SH-1 forms).

† Values for apparent $V_{\max}$ ($V_{\max}^{\text{NAD}^+}$) and corresponding apparent K_m for NAD^+ ($K_m^{\text{NAD}^+}$) given in parts III and V respectively of this Table are from non-linear regression analysis of activity data obtained at various [NAD^+] (up to 5 mM for BDH-MPL and up to 20 mM for BDH-II, -III and -IV) (results not shown). (*R*)-BOH, added as the racemic mixture, was either 10 mM for BDH:MPL at 40 mM total Na^+ or 100 mM for all samples at 240 mM Na^+ .

‡ For BDH-II and BDH-III the apparent $V_{\max}$ values obtained from activity versus [NAD^+], i.e. $V_{\max}^{\text{NAD}^+}$, significantly underestimate true $V_{\max}$ values, since the [(*R*)-BOH] used in these studies (100 mM, less than half the apparent K_m^{BOH} for these derivatives) was not saturating. For BDH-I and BDH-IV, apparent $V_{\max}$ values obtained by varying [(*R*)-BOH] or [NAD^+] are comparable, since for both these the fixed [substrate] approached saturation (more than 10-fold the apparent K_m values).

(Figure 1) were similar to the original BDH:MPL, confirming that regeneration of SH-1 restored enzymic function.

Apparent K_m and $V_{\max}$ of BDH:MPL and thiol-derivatized forms of the enzyme

The effects of modification of SH-1 and/or SH-2 of BDH:MPL upon enzymic function measured at high salt concentration (240 mM Na^+) are summarized in Table 1. For BDH:MPL, the high salt reduces enzymic activity to about half its value at 40 mM Na^+ , with little effect on K_m values; the apparent K_m^{BOH} is somewhat greater at higher salt, while apparent $K_m^{\text{NAD}^+}$ is essentially unaffected by salt concentration in this range (Table 1). For DM(SH-1) modified forms of the enzyme (BDH-II and -III), the activity is decreased markedly relative to the control BDH:MPL. The low activity of both BDH-II and BDH-III is enhanced somewhat (approx. 3-fold) by high substrate. While reduction of BDH-II with DTT restored activity (see above), only partial activity (about 40%) is recovered from the doubly derivatized BDH-III when SH-1 is regenerated by treatment with DTT, yielding BDH-IV (Figure 1, lower left panel).

The enzymic activity of BDH-MPL and three derivatized forms of the enzyme (BDH-II, -III and -IV, see Scheme 1) was measured as a function of either [(*R*)-BOH] (see Figure 2) or [NAD^+] (results not shown) to determine apparent Michaelis constants (K_m) and apparent $V_{\max}$ values. Unlike BDH:MPL (BDH-I) (results not shown) and MAL(SH-2)BDH:MPL (BDH-IV) (Figure 2, upper curve), the SH-1-modified forms of the enzyme (BDH-II and -III) do not reach a plateau in activity in

this range of substrate concentration, indicating a marked increase in K_m^{BOH} . For SH-1-modified forms of the enzyme (BDH-II and -III), there is an ~ 100 -fold increase in the apparent K_m^{BOH} and a significant increase (5–7-fold) in the apparent $K_m^{\text{NAD}^+}$ compared with BDH-I (Table 1). The values of apparent K_m^{BOH} calculated for DM(SH-1) modified forms (BDH-II and -III), 200 and 260 mM respectively, are approximate, since they exceed the maximal concentration of (*R*)-BOH (100 mM) that could be used while maintaining total [Na^+] at 240 mM. This experimental limitation may also contribute to the increase in apparent $K_m^{\text{NAD}^+}$ for the SH-1-modified forms of the enzyme, i.e., the apparent $K_m^{\text{NAD}^+}$ was determined with an [(*R*)-BOH] (100 mM) less than the apparent K_m^{BOH} for these forms of the enzyme, whereas for BDH-I and BDH-IV this concentration of substrate is essentially saturating. For the enzyme modified only at SH-2 (BDH-IV), there is no change in the apparent K_m^{BOH} compared with the control (BDH-I), but the apparent $K_m^{\text{NAD}^+}$ is increased by about 2-fold and apparent $V_{\max}$ reduced to about 40% of the control. The increase in $K_m^{\text{NAD}^+}$ for BDH-IV may reflect a somewhat reduced affinity of the SH-2-modified enzyme for NAD^+ , since, in this case, the substrate concentration was well in excess of the K_m^{BOH} .

Derivatization of SH-1, the more rapidly reacting thiol group, leads to more profound alteration of enzymic function than does derivatization of SH-2 (Table 1). The retention of even a small fractional activity for SH-1-derivatized BDH and the enhanced catalysis observed at high substrate concentrations is consistent with: (1) the fully derivatized enzyme having a greatly reduced $V_{\max}$; and (2) previous studies indicating that SH-1 is located

Figure 2 Enzymic activity of three alkylated BDH:MPL preparations as a function of [(*R*)-3-BOH]

The activity of three derivatives of BDH [DM(SH-1)BDH:MPL (BDH-II) (□), DM(SH-1)MSL(SH-2)BDH:MPL (BDH-III) (■) and MAL(SH-2)BDH:MPL (BDH-IV) (*)] (Pathway 2, Scheme 1) were measured, in the absence of DTT, in assay medium with 240 mM total Na⁺, 20 mM NAD⁺ and various concentrations (as indicated) of (*R*)-BOH, added as the racemic mixture, (*R,S*)-BOH. Activity is expressed as the percentage of the activity of dialysed BDH:MPL (79 μmol/min per mg) measured in 40 mM Na⁺ with 5 mM NAD⁺ and 20 mM (*R,S*)-BOH (condition A, Figure 1). The apparent $V_{\max}$ and apparent K_m values (K_m^{BOH} and $V_{\max}^{\text{BOH}}$) were obtained by fitting the data to a rectangular-hyperbolic function (continuous curves); mean values from several determinations are given in Table 1.

near the catalytic centre, but is not chemically essential for enzyme activity [28]. By contrast, modification of SH-2 (BDH-IV) results in only a modest reduction in enzymic activity (to about 30–40 % of the control) and little or no effect on K_m values (Table 1). We conclude from these studies that SH-2 is not in the catalytic centre, albeit derivatization of this site reduces the catalytic rate. The changes in K_m^{BOH} and $V_{\max}^{\text{BOH}}$ as a result of selective thiol modification imply that SH-1 is located closer to the substrate-binding site and that SH-2 is located more distal to this site.

Derivatization of SH-2 with maleimide reagents of variable size

Maleimide substituents of variable size, including (a) NEM, (b) MSL, (c) CPM and (d) FLM, as illustrated in Figure 3, were used to selectively derivatize SH-2 of BDH:MPL. For these studies, a single preparation of DM(SH-1)BDH:MPL was divided into four aliquots, each of which was derivatized with one of the four RMAL reagents. BDH enzymic activities of each preparation [DM(SH-1)R-MAL(SH-2)BDH:MPL] were then measured (Figure 4, upper panel) and after regeneration of SH-1 by treatment with dithiothreitol [RMAL(SH-2)BDH:MPL; Figure 4, lower panel]. The enzyme derivatized with MSL (Figure 4) is equivalent to BDH-III and -IV (see Figure 1, lower panels). For each doubly derivatized form of the enzyme [DM at SH-1 and RMAL at SH-2] (Figure 4, upper panel), the highest activity is with high substrate and coenzyme (condition E) and enzymic activity decreases as the size of the substituent at SH-2 increases. Reduction of the doubly derivatized forms of the enzyme with DTT (Figure 4, lower panel) results in a 10-fold or more increase in enzymic activity (assay condition A). Moreover, the pattern of relative activities within each set of activity measurements (A, C, E) changes so that condition A gives the highest activity, consistent with regeneration of the thiol at SH-1 (see Figure 1). As for the doubly derivatized preparations, the enzymic activity

Figure 3 Maleimide reagents (RMAL) of different size used to alkylate DM(SH-1)BDH:MPL at SH-2

The reagents used to investigate the effect of reagent size on the catalytic activity of BDH were NEM, MSL, CPM and FLM. The procedure for derivatization was similar to that described in Scheme 1 for DM(SH-1)BDH:MPL + MSL.

of RMAL(SH-2)BDH:MPL generally decreases as the size of the covalent substituent at SH-2 is increased. The activity of the FLM derivative is somewhat higher than that for the CPM adduct, although FLM is the larger (431 as against 406 Da). The shape, i.e. CPM versus FLM and/or electrostatic properties of each derivatizing reagent may also contribute to the effects of these derivatives on the activity of BDH. It should be noted, however, that even the largest derivatives at SH-2 of BDH have a smaller effect on enzymic activity than does derivatization of SH-1 with DM.

Thiol localization with respect to the domain structure of BDH

The recently determined primary sequence of human heart BDH (297 residues) shows that the N-terminal domain (residues 10–193) has sequence similarity to that of the family of short-chain alcohol dehydrogenases [22]. On the basis of alignment with a previously reported peptide that contained the reactive thiol [20], SH-1 is at Cys-242 [22], a residue that is conserved in the primary sequence of rat liver BDH [21]. Thus the reactive thiol of BDH appears to be in the C-terminal domain of the protein (residues 194–297). This domain is predicted to include elements that contribute to substrate specificity and PC-binding sites in this lipid-requiring dehydrogenase [22]. Previous studies have shown that: (1) alkylation at SH-1 markedly reduced the activity of BDH [17]; (2) modification of SH-1 proceeded at a lower rate in the presence of nucleotide [17,29]; (3) in the enzyme–NAD⁺ complex, half-site reactivity was conferred upon

Figure 4 Comparison of the relative activities of doubly derivatized DM(SH-1)RMAL(SH-2)BDH:MPL and DTT-reduced singly derivatized RMAL(SH-2)BDH:MPL for maleimide reagents (RMAL) of differing size

The diamide derivative DM(SH-1)BDH:MPL was prepared from BDH:MPL as described in the Materials and methods section and in Scheme 1. To aliquots of the DM(SH-1)BDH:MPL were added 50 equiv. of each of the RMALs with icons defined in Figure 3. The four reactions were initiated concurrently and, after 4 h, each reaction mixture was divided into two portions, one for immediate enzyme assay of the doubly derivatized enzyme and the other for treatment with 50 mM DTT to regenerate SH-1. The double derivatives DM(SH-1)RMAL(SH-2)BDH:MPL (upper panel) were assayed under the high $[NAD^+]$ conditions (ACE) (see Figure 1) immediately after the 4 h derivatization reaction. After an additional 4 h incubation at 4 °C, the DTT-treated portions of each reaction mixture (lower panel) were analysed by using the same set of three assay conditions. Activities are plotted as the percentage of dialysed BDH:MPL control activity (58 $\mu\text{mol}/\text{min}$ per mg) under condition A.

SH-1, whereas in the absence of NAD^+ the stoichiometry of derivatization was one SH-1 per BDH protomer [19]; (4) alkylation of SH-1 perturbed energy transfer from an intrinsic tryptophan of BDH to bound NADH acceptor [19]; and (5) derivatization of the enzyme at SH-1 with reagents of variable size resulted in diminishing activity correlated with the size of the derivatizing reagent. Thus derivatization of SH-1 alters both the substrate- and cofactor-binding sites, with the reduction in enzymic activity being approximately proportional to the size of the derivatizing reagent. The effect of cyanylation of SH-1 of BDH on the kinetic parameters of the enzyme was to increase apparent K_m^{BOH} and $K_m^{\text{acetoacetate}}$ 10-fold and $K_m^{\text{NAD}^+}$ and K_m^{NADH} about 2-fold [26]. We have now shown that derivatization of SH-1 with a larger reagent, diamide, has an even larger effect on the apparent K_m^{BOH} than does cyanylation (100-fold versus 10-fold increase), indicating that SH-1 is located at or near the substrate-

binding site and consistent with the prediction [22] that the C-terminal domain provides specificity in substrate binding.

We find only small effects of SH-1 derivatization on coenzyme binding to BDH [2-fold or 5-fold increases in the apparent $K_m^{\text{NAD}^+}$ by cyanylation [26] or diamide derivatization (Table 1)]. Thus there are limited effects of SH-1 derivatization on NAD^+ binding to BDH, although it has been shown previously that: (1) bound NAD^+ alters the reactivity of SH-1 [17,19,29]; and (2) the reactive thiol (SH-1) can be derivatized with arylazido- NAD^+ , indicating that it is near the active site [20]. Our results indicate that SH-1 is located at or near the substrate-binding site, but is not involved directly in nucleotide binding. However, since bound NAD(H) reduces the reactivity of SH-1, this thiol may reside in a pocket that is partially occluded by bound NAD(H).

The N-terminal sequence of BDH (residues 19–67) [22] appears to constitute the NAD(H)-binding site, on the basis of sequence similarity to a putative NAD(H)-binding domain (residues 6–62) identified in the X-ray structure of one of the short-chain dehydrogenases ($3\alpha,20\beta$ -hydroxysteroid dehydrogenase) [30]. Since the C-terminal domain of BDH provides substrate specificity and contains the arylazido- NAD^+ -reactive thiol (SH-1), the two domains would appear to be in close proximity in the folded (ternary or quaternary) structure [22]. In this regard the activation of BDH by PC is due in large part to PC-induced enhancement of nucleotide binding, i.e., a 50-fold decrease in the dissociation constant for NAD(H) in the presence versus the absence of PC [11,12]. Thus, although elements which constitute the PC-binding site are predicted to be in the C-terminal domain of BDH, activation of the enzyme by PC enhances nucleotide binding, the site for which is in the N-terminal domain of the protein. Spatial relationships between the sites and the mechanism for PC activation are being investigated. Spin-probe-spin-label studies have shown that SH-1 is located at least 0.9 nm (9 Å) from the surface of the phospholipid bilayer [31]. The 100-fold increase in K_m^{BOH} upon derivatization of SH-1 and minimal changes in apparent K_m^{BOH} for the SH-2 alkylated enzyme found in the studies reported here indicate that these two cysteines are in different domains of BDH. Recent studies using the spin-probe-spin-label technique to determine the locale of SH-2 with respect to the bilayer surface are consistent with this interpretation [32].

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