Reversible Modification of D-β-Hydroxybutyrate Dehydrogenase by Diamide†

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ABSTRACT: D-β-Hydroxybutyrate dehydrogenase is a lipid-requiring enzyme with a specific requirement of lecithin for function. The purified enzyme devoid of lipid (apodehydrogenase) is inactive but can be reactivated by forming a complex with phospholipid containing lecithin. We find that, of the six half cysteines present in D-β-hydroxybutyrate dehydrogenase, only two are in the reduced form and available for modification with *N*-ethylmaleimide, even after denaturation in sodium dodecyl sulfate. Diamide treatment of either the inactive apodehydrogenase or the active enzyme–phospholipid complex resulted in complete loss of enzymic activity, the apodehydrogenase being assayed after addition of phospholipid. The inactivation by diamide can be reversed by the addition of dithiothreitol with full recovery of activity. Derivatization using *N*-[^{14}C]ethylmaleimide showed that diamide modified only one sulfhydryl per enzyme monomer. The other sulfhydryl appears not to be essential for function since full activity can be restored after this sulfhydryl had been covalently derivatized with *N*-ethylmaleimide. Protein cross-linking was not observed after diamide modification of D-β-hydroxybutyrate dehydrogenase, indicating that a disulfide bridge was not formed between enzyme subunits. The diamide-modified enzyme retains the ability to bind coenzyme, NAD(H), as detected by quenching of the intrinsic fluorescence of the protein. However, resonance energy transfer from protein to bound NADH and enhancement of NADH fluorescence were not observed, indicating that diamide modification of the protein alters the nucleotide binding site. We conclude that (1) the native enzyme contains two thiol groups and two disulfide bridges per monomer, (2) diamide reacts with only one sulfhydryl which is essential for function, (3) diamide treatment does not result in cross-linking of enzyme monomers, (4) both the essential and nonessential sulfhydryls exhibit half-of-site reactivity, (5) the lability of the enzyme, referable to the essential sulfhydryl, can be protected by diamide, (6) the essential sulfhydryl is not required for binding of the coenzyme, and (7) selective derivatization of the nonessential sulfhydryl is made possible by prior modification with diamide.

D-β-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) is a lipid-requiring enzyme which has been purfied to homogeneity from beef heart mitochondria as the apodehydrogenase, i.e., the enzyme devoid of phospholipid. The apodehydrogenase is reactivated by insertion into phospholipid vesicles [for reviews see, Fleischer et al. (1974, 1983)] and is a tetramer as is the enzyme in the native mitochondrial inner membrane (McIntyre et al., 1983). Derivatization of a single essential sulfhydryl by *N*-ethylmaleimide alters the NADH binding characteristics, and in the presence of substrates or coenzyme, the essential sulfhydryl exhibits "half-site" reactivity (McIntyre et al., 1984). In this study, we characterize the modification of the enzyme with diamide.

Experimental Procedures

Some of the experimental methodology was already given by McIntyre et al. (1984); only additional procedures, pertinent to this study, are given here.

Materials. Diamide [1,1′-azobis(*N*,*N*-dimethylformamide)] was obtained from Aldrich (Milwaukee, WI) and the other reagents as described previously (McIntyre et al., 1984). All solutions were prepared in deionized water.

Assays. Assays were carried out as described (McIntyre et al., 1984) except that for measurement of D-β-hydroxybutyrate dehydrogenase activity dithiothreitol (DTT)[1] and bovine plasma albumin were omitted from the assay mixture to avoid reactivation of the diamide-treated enzyme.

Modification of D-β-Hydroxybutyrate Dehydrogenase with Diamide. The preparation of D-β-hydroxybutyrate dehydrogenase, mitochondrial phospholipid (MPL) vesicles, and the active enzyme–MPL complex (free of DTT) are as described previously (McIntyre et al., 1984). Derivatization of the enzyme with diamide was carried out in sealed vials under N_2 to minimize inactivation by air oxidation (Latruffe et al., 1980). D-β-Hydroxybutyrate dehydrogenase, as the active enzyme–MPL complex (100 μg of lipid phosphorus/mg of protein), was incubated (4 °C) at 0.2 mg of protein/mL in 5 mM Hepes, 50 mM NaCl, and 1 mM EDTA, pH 7.3. Diamide was added from a stock solution (0.1 M) in ethanol to a final concentration of 1 mM. Ethanol at 1% does not result in inactivation. The inactivation of the enzyme–MPL complex was followed with time until <1% of the original activity remained. The reaction mixture was then dialyzed for 36 h against three changes of N_2-saturated buffer (>100 volumes) containing 5 mM Hepes, 50 mM NaCl, and 1 mM EDTA (pH 7.0 at 4 °C) to remove excess diamide.

Reactivation of Diamide-Inactivated D-β-Hydroxybutyrate Dehydrogenase. The diamide-inactivated enzyme (see above) was reactivated by reduction with DTT. For routine measurement, 1 μL of 1 M DTT was added to 10 μL of enzyme–MPL complex inactivated with diamide, giving a final concentration of 100 mM DTT. Enzymic activity was measured after a 5-min incubation at room temperature (25 °C).

Estimation of the Number of Sulfhydryls Modified by Diamide. Derivatization with *N*-ethylmaleimide was used to quantitate by difference the number of sulfhydryls modified

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[1] Abbreviations: diamide, 1,1′-azobis(*N*,*N*-dimethylformamide), also called diazenedicarboxylic acid bis(dimethylamide); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid: MPL, the mixture of mitochondrial phospholipids prepared from beef heart mitochondria (Fleischer et al., 1967); NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; SDS, sodium dodecyl sulfate.

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by diamide. For the native enzyme, the DTT-free enzyme–MPL complex was derivatized with *N*-[^{14}C]ethylmaleimide, before or after treatment with diamide, as described (McIntyre et al., 1984) except that, at time intervals, 50-μL aliquots were removed and DTT was added to a final concentration of 100 mM, both to stop the reaction of *N*-ethylmaleimide and to reverse the diamide inactivation. After a 5-min incubation with the DTT, enzymic activity was measured, and the remainder of the sample was assayed for ^{14}C incorporation after acid precipitation (Latruffe et al., 1980).

For quantitation of the number of sulfhydryls modified by diamide treatment of D-β-hydroxybutyrate dehydrogenase, the enzyme was denatured in sodium dodecyl sulfate and then derivatized with *N*-[^{14}C]ethylmaleimide. The DTT-free enzyme–MPL complex, before or after treatment with diamide, was kept in a sealed ampule under a N_2 atmosphere and was denatured in sodium dodecyl sulfate, at a final concentration of 2%, by heating to 70 °C for 90 min. After the solution was cooled, *N*-ethylmaleimide was added to a 30-fold excess over D-β-hydroxybutyrate dehydrogenase. The *N*-ethylmaleimide was either ^{14}C radiolabeled or unlabeled as indicated. After a 10-h reaction time at room temperature, 100 mM DTT was added both to quench the reaction and to reduce disulfide bridges and the diamide-modified sulfhydryls. The samples were then dialyzed exhaustively vs. 0.2% sodium dodecyl sulfate to remove excess *N*-ethylmaleimide and DTT. After dialysis, a portion of the sample was counted for ^{14}C-label incorporation and the remainder reacted again with *N*-[^{14}C]ethylmaleimide for 10 h and processed as after the initial labeling reaction. Thus, the ^{14}C-label incorporation from *N*-ethylmaleimide into D-β-hydroxybutyrate, with and without diamide pretreatment, was quantitated both prior to and subsequent to reduction of the protein with DTT.

Polyacrylamide Gel Electrophoresis. The molecular size of D-β-hydroxybutyrate dehydrogenase after treatment with diamide was characterized by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, according to Laemmli (1970), using 15% polyacrylamide slab gels. Samples of diamide-treated and nontreated D-β-hydroxybutyrate dehydrogenase (as the MPL complex) were prepared as follows: Each sample (75 μL, 50 μg of protein/mL) was diluted with 25 μL of sample buffer [2 M Tris-HCl, pH 6.8, 8% sodium dodecyl sulfate, 22% glycerol (v/v), and 0.22 mg of bromophenol blue/mL] and incubated for 30 min at room temperature. A 15-μL aliquot was loaded on the gel. Electrophoresis was carried out on a Hoefer slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA) at 1.5 mW/gel with cooling and was terminated when the tracking dye (bromophenol blue) reached the bottom of the gel (4 h). Gels were silver stained according to Merril et al. (1983). Samples were also analyzed on separate gels by using the same procedure, but 10 mM DTT was included in the sample buffer.

Results

Modification Studies. In the presence of diamide, D-β-hydroxybutyrate dehydrogenase, as the active complex with MPL, is inactivated with a half-time of 7 min at 0 °C (Figure 1). The rate of inactivation is 3-fold faster at room temperature (Figure 1 and Table I). Treatment of the apodehydrogenase, which is devoid of phospholipid and inactive, results in loss of the ability to reactivate the enzyme with phospholipid (Figure 1). The diamide-modified enzyme–MPL complex is reactivated to full activity within 5 min by addition of DTT (Figure 1). Likewise, the diamide-treated apodehydrogenase is fully reactivated by DTT (not shown). When the diamide-modified enzyme complex is dialyzed to remove excess diamide, the enzyme remains inactive but can be fully reactivated with 5 mM DTT (not shown). In the presence of diamide, the amount of DTT necessary for full reactivation of the enzyme is higher (up to 100 mM) than that in the absence of diamide. The diamide-modified enzyme, after dialysis to remove excess diamide, is stable in the cold (5 °C). After several weeks, full activity was restored upon addition of DTT (not shown). Thus, diamide treatment affords complete protection of the enzyme.

Table I: Effect of Nucleotides, Substrates, and Competitive Inhibitor on Diamide Inactivation of D-β-Hydroxybutyrate Dehydrogenases[a]

additions[b]	$t_{1/2}$ (min)
none[c]	2.5 ± 0.5
NADH	2.5 ± 0.5
NAD^+	3.0 ± 0.5
DL-β-hydroxybutyrate	2.5 ± 0.5
acetoacetate	2.0 ± 0.5
2-methylmalonate	28 ± 3
NADH + DL-β-hydroxybutyrate[d]	12 ± 2[e]
NAD^+ + acetoacetate[d]	3.0 ± 0.5
NADH + 2-methylmalonate	120 ± 10
NAD^+ + 2-methylmalonate	130 ± 10

[a] The DTT-free enzyme, as the active complex with MPL, was prepared and treated with diamide (1 mM) as described in Figure 1 except that the reaction was carried out with 0.15 mg of BDH/mL in 50 mM NaCl, 5 mM Tris-HCl (pH 8.0 at 20 °C), and 1 mM EDTA and at room temperature (~26 °C). The increased temperature and pH enhance the rate of inactivation (see footnote *c*). Nucleotides (5 mM NAD^+ or NADH) and/or substrates (100 mM DL-β-hydroxybutyrate or 50 mM acetoacetate) or competitive inhibitor (50 mM 2-methylmalonate) was added 3 min prior to diamide. The residual enzymic activity was measured as a function of time after initiation of the reaction, and the $t_{1/2}$ times were obtained from plots of the logarithm of enzymic activity as a function of time (cf. Figure 1). These plots were linear in all cases to <10% of the initial activity except in the presence of NADH together with DL-β-hydroxybutyrate (see footnote *e*). The intial activity of different preparations of DTT-free enzyme–MPL complex used for these experiments varied between 120 and 147 μmol of NAD^+ reduced/(min·mg of protein). In all cases, enzymic activity was fully recovered by subsequent incubation with 100 mM DTT (cf. Figure 1). [b] The dissociation or kinetic binding constants for the various ligands are as follows: NADH, K_d = 15 μM (Gazzotti et al., 1974), K_i = 50 μM; NAD^+, K_d = 40 μM (cf. Table III), K_i = 1.7 mM; acetoacetate, K_m = 1.6 mM; D-β-hydroxybutyrate, K_m = 2.0 mM; 2-methylmalonate, K_i = 0.1 mM (Tan et al., 1973). The K_i and K_m values for nucleotides and substrates are from Nielsen et al. (1973). [c] At pH 7.3 and 0 °C, the inactivation rate is ~3 times slower with a $t_{1/2}$ of 7 ± 1 min (Figure 1). [d] These mixtures of ligands from "dead-end" or "abortive ternary" complexes with the enzyme since nucleotide and substrate are either both reduced or both oxidized. [e] With both NADH and DL-β-hydroxybutyrate together, the inactivation with diamide stopped after about 10 min of reaction. The half-time is estimated by extrapolation of the initial time points. Further additions of diamide eventually result in complete inactivation of the enzyme, indicating that the reaction stops due to depletion of diamide. Preincubation of NADH, DL-β-hydroxybutyrate, and diamide together with a catalytic amount of enzyme (0.2 μg of BDH in 100 μL) resulted in complete reduction of the diamide in 1 h since no subsequent inactivation of added BDH was obtained. In the absence of enzyme, NADH and β-hydroxybutyrate do not alter the diamide in terms its ability to modify added enzyme. Thus, in the presence of NADH and β-hydroxybutyrate, BDH, catalyzes the reduction of diamide.

The addition of nucleotides or substrates alone does not significantly affect the rate of inactivation of the enzyme–MPL complex as reflected by similar $t_{1/2}$ values (Table I). The addition of 2-methylmalonate, a competitive inhibitor with respect to D-β-hydroxybutyrate (Tan et al., 1975), gives a 10-fold longer $t_{1/2}$ value for reaction with diamide. The formation of an abortive ternary complex of the enzyme together with NAD^+ and acetoacetate does not afford protection against inactivation by diamide. In contrast, the abortive ternary complex with NADH and β-hydroxybutyrate appears to give

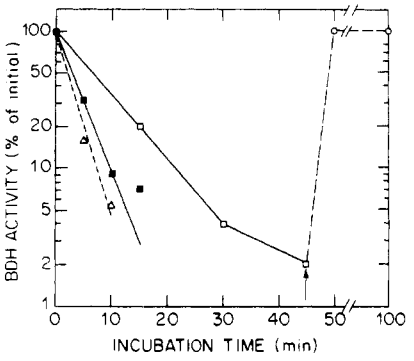

FIGURE 1: Time course of inactivation of D-β-hydroxybutyrate dehydrogenase (BDH) by diamide and reactivation with DTT. BDH was inactivated either as apodehydrogenase or as the active BDH–MPL complex. The apodehydrogenase was dialyzed at 1 mg/mL vs. 50 mM NaCl, 5 mM Hepes, and 1 mM EDTA, pH 7.3, to remove DTT and was diluted to 0.2 mg/mL (6.3 μM BDH monomer) and kept at 25 °C. Diamide (0.1 M in ethanol) was added to a final concentration of 1 mM, and aliquots were taken at the times indicated and diluted 10-fold into buffer containing MPL (150 μg of lipid phosphorus/mg of BDH), 5 mM NAD^+, and 10 mM methylmalonate; after 30-min incubation, enzymic activity was measured (see Experimental Procedures) at 37 °C (△). The initial specific activity was 115 μmol of NAD^+ reduced/(min·mg of BDH). The active BDH–phospholipid complex was formed by incubation of the apodehydrogenase with MPL (150 μg of lipid phosphorus/mg of BDH) for 2 h at room temperature to obtain optimal activation [150 μmol of NAD^+ reduced/(min·mg of BDH) at 37 °C]. After dialysis vs. 50 mM NaCl, 5 mM Hepes, and 1 mM EDTA, pH 7.3, to remove DTT, the enzyme was diluted in the same buffer to 0.2 mg/mL (6.3 μM BDH monomer, assuming a monomer molecular weight of 31 500). The initial specific activity after dialysis was 147 μmol of NAD^+ reduced/(min·mg of protein). Diamide (0.1 M in ethanol) was added to a final concentration of 1 mM, and aliquots were taken at the times indicated and added directly to assay medium to measure enzymic activity. The reaction of BDH–MPL with diamide was carried out either at 0 (□) or at 25 °C (■). The half-times for inactivation ($t_{1/2}$) under different conditions are given in Table I. After a 45-min incubation of the BDH–MPL complex with diamide at 0 °C (residual activity was 2% of initial value), 1 M DTT was added to give a final concentration of 100 mM DTT (arrow), and reactivation of enzymic function was measured at time intervals (○). The apodehydrogenase was also reactivated with DTT in like manner (not shown). There was no detectable loss of activity during 3 h of incubation in the absence of diamide. After reactivation of the diamide-treated enzyme with DTT, activity remained constant for several days as for the control which was not diamide treated.

some protection (4–5-fold). However, under these conditions, the inactivation rate appears to be influenced by enzymic reduction of the diamide since complete inactivation was obtained only after several additions of diamide (see footnote *e* of Table I). The formation of a dead-end complex of active enzyme–MPL together with both nucleotide (NAD^+ or NADH) and 2-methylmalonate results in a dramatic decrease in the rate of inactivation by diamide ($t_{1/2}$ is 50-fold greater) (Table I).

When the diamide-modified D-β-hydroxybutyrate dehydrogenase is treated with 5- or 30-fold molar excess of *N*-[^{14}C]ethylmaleimide for up to 2 h, activity is fully restored upon subsequent addition of DTT (Figure 2). In contrast, the native enzyme is inactivated with 5-fold excess of *N*-ethylmaleimide with concomitant incorporation of 1 equiv of ^{14}C label into the protein as reported previously (Latruffe et al., 1980; McIntyre et al., 1984). After prolonged incubation, approximately 2 mol of ^{14}C label is incorporated per enzyme monomer (cf. Table II). For the native enzyme, approximately 1 equiv is incorporated rapidly with concomitant loss of activity, and subsequent derivatization proceeds more slowly. For the diamide-modified enzyme, ^{14}C label is also incorporated into the protein but at significantly reduced levels for

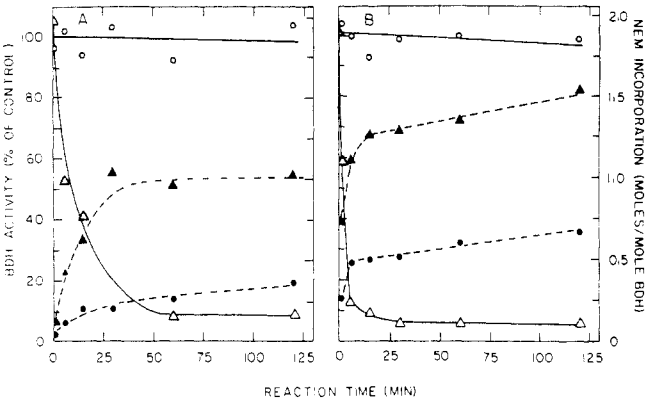

FIGURE 2: Time course of derivatization and inactivation of diamide-treated D-β-hydroxybutyrate dehydrogenase (BDH) by *N*-ethylmaleimide (NEM). Inactivation by NEM was measured as loss of BDH activity that is not recovered by DTT treatment since diamide-inactivated BDH is reactivated by DTT (Figure 1). The BDH–MPL complex was prepared and inactivated by treatment with diamide (1 mM) for 2 h as described in Figure 1. After dialysis vs. 50 mM NaCl, 5 mM Hepes, and 1 mM EDTA, pH 7.0, to remove excess diamide, the enzyme was diluted to 0.14 mg of protein/mL in the same buffer. [^{14}C]NEM was added to either a 5-fold (A) or 30-fold (B) excess per BDH monomer and incubated on ice. Aliquots were removed at time intervals as indicated, made 100 mM in DTT (both to stop the NEM reaction and to reverse the diamide inactivation), and (after a 10-min incubation) assayed for BDH activity (open symbols) and incorporation of ^{14}C label (filled symbols). Data are shown for the control enzyme (△, ▲) (initial specific activity of 135) and for diamide-treated enzyme (○, ●) (initial loss of specific activity with diamide to <1% of control and reactivated with DTT to a specific activity of 132). Incubation of the enzyme with a 30-fold excess of [^{14}C]NEM for 18 h resulted in incorporation of 2.0 ± 0.2 and 1.0 ± 0.1 equiv of ^{14}C label for native and diamide-inactivated BDH, respectively.

both a 5-fold and 30-fold excess of reagent (parts A and B of Figure 2, respectively) while the protein remains fully reactivatable by subsequent DTT treatment. Activity is not lost; albeit 0.5 equiv of ^{14}C label is incorporated rapidly (10 min) followed by a slow derivatization with incorporation of only 0.15 additional equivalents during the remaining 110 min of the experiment. It should be noted that with 30-fold excess of *N*-[^{14}C]ethylmaleimide, the incorporation of ^{14}C label into the protein is distinctly biphasic for both the native and the diamide-treated enzyme (Figure 2B). Since, in the diamide-treated enzyme, only one sulfhydryl per monomer is available for derivatization (see below), this derivatization with *N*-ethylmaleimide appears to exhibit a kind of "half-of-site" reactivity as has been found previously for the essential sulfhydryl (McIntyre et al., 1984).

For diamide-modified D-β-hydroxybutyrate dehydrogenase treated with 30-fold excess *N*-ethylmaleimide for up to 2 h, essentially all activity can be recovered by treatment with DTT even though >0.6 equiv of ^{14}C label had been incorporated (Figure 2B). However, when derivatization of the diamide-treated enzyme was carried out for a prolonged time with 100-fold excess of *N*-ethylmaleimide, a slow, irreversible inactivation ($t_{1/2}$ > 150 min) was observed; i.e., not all activity could be recovered by subsequent incubation with excess DTT (not shown). This inactivation appears to result from derivatization of a group other than a sulfhydryl since (1) the rate of inactivation is very slow for the reaction of a sulfhydryl with a large excess of *N*-ethylmaleimide and (2) the irreversible inactivation rate was similar in the presence of methylmercury, a reversible sulfhydryl reagent.

The data in Figure 2 show that inactivation of D-β-hydroxybutyrate dehydrogenase by diamide decreases the subsequent incorporation of *N*-ethylmaleimide into the protein.

Table II: Quantitation of Sulfhydryls in D-β-Hydroxybutyrate Dehydrogenase Modified by Diamide prior to Denaturation in Sodium Dodecyl Sulfate[a]

sequence of reactions	[^{14}C]NEM incorporated (mol/mol of BDH)	
	no diamide pretreatment	diamide treated
(1) [^{14}C]NEM[b]	1.9	1.0
(2) DTT reduction, [^{14}C]NEM	5.5	5.5
(3) NEM, DTT reductions, [^{14}C]NEM	3.5	4.7

[a] D-β-Hydroxybutyrate dehydrogenase (BDH), as the active complex with MPL vesicles, was prepared free of DTT as described in Figure 1. One sample was treated with 1 mM diamide for 2 h and excess diamide removed by dialysis. A second sample was not treated with diamide. Both diamide-treated and control samples were denatured by heating at 70 °C for 90 min in 2% sodium dodecyl sulfate (for details see Experimental Procedures), and each sample was then divided into three portions (numbers 1, 2, and 3). (1) An aliquot of each was treated for 10 h with *N*-[^{14}C]ethylmaleimide ([^{14}C]NEM) 30-fold excess over BDH and incorporation of ^{14}C label quantitated (the first entry in the table). (2) An aliquot of each was incubated for 1 h with 100 mM DTT both to reduce the diamide-modified sulfhydryls and to reduce disulfide bridges, then dialyzed, and labeled (10 h) with [^{14}C]-NEM, at 30-fold excess over BDH (second entry in the table). (3) An aliquot of each was treated (10 h) with unlabeled NEM (30-fold excess), then reduced with 100 mM DTT (to quench the NEM reaction and to reduce the diamide-modified sulfhydryls and the disulfide bridges), dialyzed exhaustively, and then reacted with [^{14}C]NEM (10 h). After dialysis, ^{14}C-label incorporation was quantitated to measure the number of sulfhydryls that become exposed by reduction of the denatured enzyme with DTT (last entry in the table). In each case (1, 2, and 3), incorporation of ^{14}C label was quantitated after exhaustive dialysis vs. 0.2% sodium dodecyl sulfate. All reactions and dialyses were carried out under a N_2 atmosphere. Preparation of DTT-free enzyme and diamide treatment were carried out at 0 °C. All other procedures were performed at ambient temperature (23–25 °C). [b] In the absence of sodium dodecyl sulfate, the incorporation of [^{14}C]NEM (30 mol/mol of BDH for 18 h) into BDH was 2 and 1 equiv/BDH subunit for native and diamide-modified BDH, respectively (cf. legend of Figure 2).

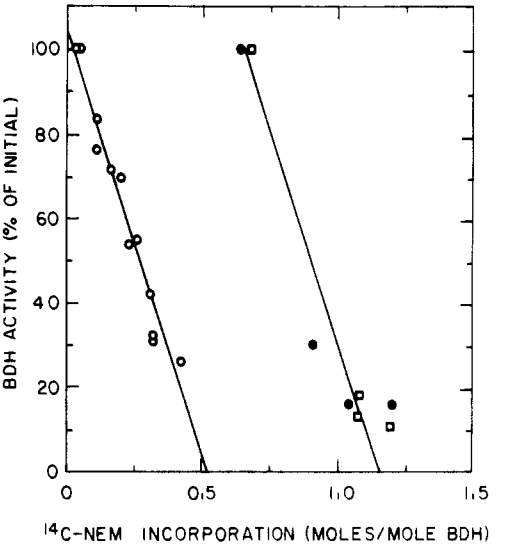

FIGURE 3: Stoichiometry of inactivation of D-β-hydroxybutyrate dehydrogenase (BDH) by *N*-ethylmaleimide (NEM). BDH, as the MPL complex, was inactivated with 1 mM diamide for 2 h, dialyzed to remove excess diamide, and then incubated with either 1 mM unlabeled NEM (○) or 1 mM [^{14}C]NEM (●, □) at 30 mol/mol of BDH subunit as described in Figure 2. After 2 h DTT was added to 100 mM (to stop the NEM reaction and reverse the diamide inactivation), and the samples were dialyzed 3 times vs. 100 volumes of 50 mM NaCl, 5 mM Hepes, and 1 mM EDTA, pH 7.0, to remove NEM and DTT. The samples were then inactivated with [^{14}C]NEM at either 5 (○, ●) or 30 (□) mol/mol of BDH subunit and assayed for both BDH activity and incorporation of ^{14}C label as a function of time after NEM addition to determine the inactivation stoichiometries shown. The specific activities of BDH [μmol of NAD^+ reduced/(min·mg of BDH) at 37 °C] at the various stages of the procedure were as follows: initial value, 147; after first NEM treatment and reactivation with DTT, 120 (○) or 132 (●, □); prior to inactivation by [^{14}C]NEM, 110 (○) or 120 (●, □). Lines were drawn for each set of data [(○) and (●, □)] according to the method of least squares. Slopes are 0.5 ± 0.1 for (○) and 0.5 ± 0.2 for (●, □).

Prolonged incubation of the native and diamide-inactivated enzyme with a 30-fold excess of *N*-[^{14}C]ethylmaleimide results in incorporation of approximately 2 and 1 mol of ^{14}C label/mol of enzyme monomer, respectively (cf. legend of Figure 2). Similar results are obtained by using the denatured enzyme without reduction of DTT. That is, approximately 2 mol of ^{14}C label is incorporated per mol of enzyme monomer, and only 1 mol is incorporated for the diamide-inactivated enzyme (entry 1, Table II). When the enzyme is denatured and reduced with DTT 5.5 equiv of ^{14}C label can be incorporated from *N*-[^{14}C]ethylmaleimide. The diamide-treated enzyme, which is denatured and reduced with DTT, gives the same stoichiometry (entry 2, Table II). In the absence of SDS, only two sulfhydryls are accessible for derivatization with NEM (Latruffe et al., 1980; Figure 2). We interpret these results to mean that of the six cysteines in D-β-hydroxybutyrate dehydrogenase only two exist in the reduced form as sulfhydryls in the native enzyme. The remaining four, which can be labeled with *N*-ethylmaleimide only after reduction with DTT in the presence of sodium dodecyl sulfate, are present as disulfide bridges. Treatment of D-β-hydroxybutyrate dehydrogenase with diamide results in one of the two sulfhydryls per monomer becoming inaccessible to derivatization with *N*-ethylmaleimide. The modified sulfhydryl can be regenerated by subsequent reduction with DTT (entries 1 and 3, Table II).

After the diamide-modified and *N*-ethylmaleimide-treated D-β-hydroxybutyrate dehydrogenase has been reactivated with DTT, it can subsequently be inactivated with *N*-ethylmaleimide. The sulfhydryl that is regenerated by reduction with DTT is essential for the activity of the enzyme. The inactivation stoichiometry is 0.5 mol of *N*-[^{14}C]ethylmaleimide label incorporated per enzyme monomer (Figure 3). This value is obtained whether the diamide-modified enzyme is pretreated with unlabeled *N*-ethylmaleimide or with *N*-[^{14}C]ethylmaleimide prior to reactivation with DTT. In contrast, the native enzyme is inactivated with a stoichiometry of one although in the presence of ligands (nucleotide or substrate) an inactivation stoichiometry of 0.5 is also obtained (McIntyre et al., 1984).

Nucleotide Binding Studies. The binding of NADH to the D-β-hydroxybutyrate dehydrogenase–MPL complex, both before and after modification with diamide, was studied by using fluorescence to measure NADH binding (Table III). Both native enzyme and diamide-inactivated enzyme bind NADH. The dissociation constants for NADH binding are similar for native and diamide-inactivated enzyme (Table III) although the magnitude of the quenching of the intrinsic fluorescence of diamide-inactivated enzyme was significantly smaller (by nearly a factor of 2) than for the unmodified enzyme (not shown). Neither fluorescence energy transfer (λ_{ex} 290, λ_{em} 455 nm) nor enhancement of NADH fluorescence (the ratio of NADH fluorescence in the presence vs. in the absence of enzyme) (λ_{ex} 340 nm, λ_{em} 455 nm) (cf. McIntyre et al., 1984) was observed for NADH bound to the diamide-modified enzyme as compared with the unmodified enzyme which gave signals that were higher than those for lipid alone (not shown). The binding of NAD^+ to D-β-hydroxybutyrate dehydrogenase both before and after modification with diamide

Table III: Effect of Diamide Treatment of D-β-Hydroxybutyrate Dehydrogenase on Nucleotide Binding[a]

ligand	dissociation constant (K_d) (μM) native	diamide treated
NADH	20 ± 5	25 ± 5
NAD^+	40 ± 10	90 ± 10

[a] The binding of nucleotides to D-β-hydroxybutyrate dehydrogenase (BDH), as the complex with MPL, for either native or diamide-modified complex was measured by quenching of intrinsic protein fluorescence. The active BDH–MPL complex was prepared, dialyzed to remove DTT, and inactivated by treatment with 1 mM diamide for 2 h (cf. Figure 1). The samples were then dialyzed 3 times against 100 volumes of 50 mM NaCl, 5 mM Hepes, and 1 mM EDTA, pH 8.0, and diluted to 40 μg of BDH/mL. Each sample was titrated with NADH or NAD^+, and quenching of the intrinsic protein fluorescence (λ_{ex} 290 nm, λ_{em} 340 nm) was measured. For the BDH–MPL complexes, background signal due to lipid alone (initial value 35) was subtracted. The fluorescence of the samples prior to addition of NADH was 518 (arbitrary units) for both control and diamide-treated BDH. The values for the dissociation constant are average of three sets of data in which the K_d was determined from the slope/intercept of the y axis of the double-reciprocal plots (one/quenching vs. one/nucleotide) corrected both for quenching at the excitation wavelength (290 nm) and for protein dilution during the titration.

was studied by the quenching of intrinsic fluorescence upon addition of NAD^+. The binding of NAD^+ to the diamide-treated enzyme appears to be somewhat weaker (~2-fold higher K_d) than binding of NAD^+ to the native enzyme (Table III).

Absence of Subunit Association with Diamide Treatment. Diamide is known to oxidize two adjacent sulfhydryls (vicinal dithiols) to form a disulfide bridge (Stiggall et al., 1979; Sanadi, 1982). In this study, we tried to detect whether diamide treatment of D-β-hydroxybutyrate dehydrogenase resulted in formation of a disulfide bridge between two monomers. Both native and diamide-modified D-β-hydroxybutyrate dehydrogenase were subjected to denaturation with sodium dodecyl sulfate and polyacrylamide gel electrophoresis under nonreducing conditions. A sample of diamide-modified enzyme, subsequently reactivated with DTT (followed by dialysis), was also analyzed. To ensure that no reducing equivalents were present in the samples or buffer, fresh reagents were used throughout, and in some cases, *N*-ethylmaleimide was also added to the sample buffer. Both native and diamide-modified enzyme exhibited the same staining pattern with either Coomassie brilliant blue or silver staining (not shown), i.e., essentially a single band referable to the monomer (~31 kdaltons). There was no indication that diamide modification of D-β-hydroxybutyrate dehydrogenase resulted in cross-linking to form dimers or higher oligomers.

Discussion

The studies reported here show that D-β-hydroxybutyrate dehydrogenase, either as the apodehydrogenase or as the enzyme–MPL complex, can be reversibly inactivated by treatment with diamide. The enzyme, modified with diamide, has no activity but can be fully reactivated by subsequent reduction with DTT. The modification of D-β-hydroxybutyrate dehydrogenase by diamide treatment contrasts with inactivation by aerobic oxidation where activity is not recovered by DTT treatment (Latruffe et al., 1980). The diamide-modified enzyme is a stable form in that it can be stored for several weeks in the absence of reducing agent with subsequent full recovery of activity upon reduction with DTT. Since diamide modifies the single essential sulfhydryl (see below), this sulfhydryl appears to be responsible for the lability of the enzyme. Diamide treatment is an effective procedure for protecting the labile sulfhydryl which is essential for function.

In both native and denatured D-β-hydroxybutyrate dehydrogenase, two sulfhydryls per monomer can be derivatized with *N*-ethylmaleimide. Pretreatment of the enzyme with diamide blocks the labeling of only 1 equiv of the sulfhydryls. From this, we conclude that diamide modifies one sulfhydryl per monomer, and this modification results in quantitative loss of activity. We were not able with *N*-ethylmaleimide to obtain quantitative derivatization of the other sulfhydryl in the diamide-treated native enzyme; only 0.65 equiv of ^{14}C label was incorporated without loss of activity; i.e., selective derivatization of the nonessential sulfhydryl is obtained. With higher amounts of reagent, reaction at a group other than a sulfhydryl appears to occur. The selective derivatization of the nonessential sulfhydryl in the diamide-treated enzyme is similar to that afforded by derivatization of the native enzyme in the presence of NAD^+ together with methylmalonate (McIntyre et al., 1984) except that, in the latter case, higher molar ratios of *N*-ethylmaleimide to enzyme are required. Further, with the native enzyme, protected by NAD^+ and methylmalonate, derivatization of the nonessential sulfhydryl occurs rapidly so that the kinetics could not readily be measured and are complicated by the subsequent derivatization of the essential sulfhydryl; i.e., the protection is a kinetic effect. By contrast, diamide protection involves covalent modification (albeit reversible by reduction) that allows selective derivatization of only the nonessential sulfhydryl by *N*-ethylmaleimide. This derivatization of the nonessential sulfhydryl, which is not modified by diamide treatment, is distinctly biphasic (Figure 3) and indicative of half-of-site reactivity. However, *N*-ethylmaleimide derivatization of the sulfhydryl that is not protected by diamide does not lead to loss of enzyme activity since >95% of the activity could be recovered by DTT treatment of the diamide-inactivated and *N*-ethylmaleimide-derivatized enzyme. Thus, the sulfhydryls (four per tetramer) that are not modified by diamide appear not to be essential for function. In contrast, the single sulfhydryl per monomer of D-β-hydroxybutyrate dehydrogenase, which is modified by diamide and regenerated by DTT treatment, is essential for function. Not only is the diamide-modified enzyme inactive, but subsequent selective derivatization of the diamide-sensitive sulfhydryl with *N*-ethylmaleimide results in quantitative irreversible inactivation of the enzyme. The inactivation stoichiometry of 0.5 is the same as has been observed for inactivation of D-β-hydroxybutyrate dehydrogenase by *N*-ethylmaleimide in the presence of nucleotide or substrate (McIntyre et al., 1984).

Although diamide-modified D-β-hydroxybutyrate dehydrogenase is not functional in catalysis, it retains its ability to bind nucleotide. For NADH binding, the dissociation constant is similar in native and diamide-inactivated enzyme. The binding of NAD^+ is somewhat weaker after diamide treatment (K_d increases approximately 2-fold). The differential effect of diamide treatment of the enzyme on NAD^+ and NADH binding likely reflects different geometry of the oxidized and reduced coenzymes in the nucleotide binding site. The nucleotide binding can be measured by quenching of the protein fluorescence upon titration with nucleotides, even though the magnitude of quenching is about half that observed for the active enzyme. Since the initial intrinsic protein fluorescence is similar for the active and diamide-modified preparations, it would appear that the environment of the chromophores, which contribute to the fluorescence, is not altered by diamide treatment of the enzyme in contrast to derivatization by *N*-ethylmaleimide (McIntyre et al., 1984).

Diamide treatment likely results in a change in orientation or position of the nicotinamide moiety of the bound nucleotide such that less quenching of the intrinsic fluorescence is observed. As with the enzyme inactivated with *N*-ethylmaleimide, both fluorescence energy transfer from protein to bound NADH and enhancement of NADH fluorescence are lost in the diamide-inactivated enzyme. We conclude from the fluorescence studies that the single sulfhydryl per monomer that is modified by diamide treatment is in close proximity to the NADH binding site although the enzyme inactivated by diamide retains the partial function of nucleotide binding albeit with somewhat different characteristics. Thus, NADH binding, which is a necessary first step in the ordered sequential reaction mechanism (Nielsen et al., 1973), is not of itself sufficient for catalysis.

In our earlier studies (Latruffe et al., 1980), we found two sulfhydryls were derivatized by *N*-ethylmaleimide per monomer of the native enzyme. One of the sulfhydryls was found to be essential for function and exhibited half-site reactivity (McIntyre et al., 1984). In the studies reported here, we convincingly show that there are only two accessible sulfhydryls in the native enzyme. The remainder of the sulfhydryls are not exposed by denaturation with sodium dodecyl sulfate when DTT is first removed by exhaustive dialysis. Four additional sulfhydryls can be obtained only after reduction of the denatured enzyme, with DTT yielding a total label incorporation approximating six cysteines. We conclude that four of these cysteines are linked in disulfide bridges (two per enzyme monomer) and thus are inaccessible to *N*-ethylmaleimide derivatization in either the native or denatured enzyme.

In an earlier study, it was reported that diamide inactivates D-β-hydroxybutyrate dehydrogenase in submitochondrial vesicles with a reaction order of unity (Phelps & Hatefi, 1981). Since this previous study was carried out for the enzyme in the mitochondrial membrane, chemical modification resulting from diamide treatment could not be quantitated. The results reported here with the purified enzyme demonstrate that inactivation by diamide results in the derivatization of one sulfhydryl equivalent per monomer. In contrast with the previous study, we find essentially no protection with NAD(H). We observe protection against modification by diamide only in the presence of the competitive inhibitor 2-methylmalonate either in the presence or in the absence of nucleotides but not by substrates. The specific protection by 2-methylmalonate may suggest that it binds to the enzyme in a different orientation than the substrates. The effects of the different ligands on the rate of modification by diamide are similar to their effects on inactivation by *N*-ethylmaleimide (McIntyre et al., 1984) although NADH affords protection against inactivation by *N*-ethylmaleimide but no protection against modification by diamide. The differences probably arise from the different experimental conditions used in the two studies.

One of the earliest reports of the biological effects of diamide was the intracellular oxidation of glutathione to the disulfide (Kosower et al., 1969). On the basis of these observations, it has been assumed that the reaction of proteins with diamide is limited to vicinal dithiols. Indeed, some recent observations tend to support this view in that diamide treatment of red cells results in disulfide formation and cross-linking of proteins (Davies & Palek, 1982). Diamide inactivation of mitochondrial coupling factor B has been reported to be due to the oxidation of a vicinal dithiol (Stiggall et al., 1979; Sanadi, 1982). By contrast, the results reported here for D-β-hydroxybutyrate dehydrogenase suggest that modification by diamide occurs without reaction of a vicinal dithiol in that (1) only one sulfhydryl equivalent per monomer is modified by diamide treatment and (2) cross-linking of subunits is not observed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, ruling out formation of an intersubunit disulfide bridge. A possible interpretation of our results is that diamide may not react with D-β-hydroxybutyrate dehydrogenase in a classical fashion; i.e., inactivation may not be the result of oxidation of a vicinal dithiol but could result from the formation of a bridge involving only a single sulfhydryl and another group, e.g., histidine or lysine, or, more likely, an adduct with the essential sulfhydryl, e.g., the formation of a thiol ester type product. The single sulfhydryl per monomer of D-β-hydroxybutyrate dehydrogenase that is modified by diamide treatment is essential for enzymic function of the enzyme. However, although it must be close to the active site, it seems that the thiol is not involved directly in nucleotide binding, since the diamide-treated enzyme still binds nucleotides.

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Registry No. NADH, 58-68-4; NAD, 53-84-9; D-β-hydroxybutyrate dehydrogenase, 9028-38-0; diamide, 10465-78-8.

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