Half-Site Reactivity of an Essential Thiol Group of D-β-Hydroxybutyrate Dehydrogenase†

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ABSTRACT: D-β-Hydroxybutyrate dehydrogenase is a lipid-requiring enzyme, which is a tetramer both in the mitochondrial inner membrane and as the purified enzyme reconstituted with phospholipid. For the active enzyme–phospholipid complex in the absence of ligands, we previously found that reaction with *N*-ethylmaleimide (at 5 mol/mol of enzyme subunit) resulted in progressive loss of enzymic activity with an inactivation stoichiometry of 1 equiv of sulfhydryl derivatized per mole of enzyme and a maximum derivatization of 2 equiv [Latruffe, N., Brenner, S. C., & Fleischer, S. (1980) *Biochemistry 19*, 5285–5290]. We now find, in the presence of nucleotide or substrate, that the rate of inactivation is significantly reduced, which indicates that these ligands afford protection of the essential sulfhydryl. Further, in the presence of ligands, the inactivation stoichiometry is 0.5, consistent with half-of-the-site reactivity of the essential sulfhydryl. Thus, at a low ratio of *N*-ethylmaleimide to enzyme, nucleotide or substrate affords essentially complete protection of the nonessential sulfhydryl from derivatization. The binding characteristics of NADH to both the native and *N*-ethylmaleimide-derivatized enzyme have been compared by fluorescence spectroscopy. Quenching of intrinsic tryptophan fluorescence of the protein shows that the enzyme, derivatized with *N*-ethylmaleimide either in the absence or in the presence of NAD^+, binds NADH but with a reduced K_d (~50 μM as compared with ~20 μM for native enzyme). However, a critical change has occurred in that resonance energy transfer from protein to bound NADH, observed in the native enzyme, is abolished in the *N*-ethylmaleimide-derivatized enzyme. We conclude that the essential sulfhydryl exhibits half-site reactivity and is located at or close to the NAD(H) binding site although it is not necessarily involved in the catalytic mechanism.

D-β-Hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate:NAD^+ oxidoreductase, EC 1.1.1.30) is a lipid-requiring enzyme with a specific requirement of lecithin for enzymic activity [for reviews, see Fleischer et al. (1974, 1983)]. The enzyme devoid of phospholipid (apodehydrogenase) has been purified to homogeneity and exhibits a single sharp band by polyacrylamide gel electrophoresis in sodium dodecyl sulfate or acid–urea systems (Bock & Fleischer, 1975). It is inactive but can be made functional by forming an active enzyme–phospholipid complex. The purified enzyme has a single N-terminal amino acid sequence with 1 equiv per monomer (Brenner et al., 1979). The enzyme both in the mitochondrial membrane and as the purified apodehydrogenase reconstituted into phospholipid vesicles is a tetramer (McIntyre et al., 1983).

In earlier studies, we reported that D-β-hydroxybutyrate dehydrogenase contains, per monomer, a single essential arginine (Fleer & Fleischer, 1983) and two thiol groups of which one is essential for enzymic activity (Latruffe et al., 1980). We now find that addition of substrate and coenzyme, either separately or together, modifies the reactivity of the sulfhydryl moieties to *N*-ethylmaleimide derivatization so that complete inactivation is obtained with a stoichiometry of 0.5, i.e., half-site reactivity. A preliminary report of portions of this work has been presented (McIntyre et al., 1982).

Experimental Procedures

Materials. Lithium bromide, from Fisher (Pittsburg, PA), was prepared as a 4 M stock solution and purified by filtration through a 0.22-μm filter. 2-Methylmalonate and acetoacetate were from Aldrich (Milwaukee, WI). DL-β-Hydroxybutyrate (sodium salt), *N*-ethylmaleimide, L-tryptophyl-L-alanine, and bovine serum albumin (fraction V, powder) were from Sigma Chemical Co. (St. Louis, MO). DTT[1] and NAD^+ were obtained from Chemical Dynamics Corp. (South Plainfield, NJ). Chromatopure-grade NADH, from P-L Biochemicals (Milwaukee, WI), was used for binding studies. *N*-[^{14}C]Ethylmaleimide, labeled in the C-1 of the ethyl moiety, was obtained either from New England Nuclear (Boston, MA) (specific radioactivity 2.7 mCi/mmol) or from Research Products International Corp. (Mount Prospect, IL) (4.1 mCi/mmol) and was stored in pentane under N_2 at −20 °C. Hepes was from Calbiochem (San Diego, CA). Bovine plasma albumin solution, from Armour Pharmaceutical Co. (Chicago, IL), was used as the protein standard. All other chemicals were reagent grade. Aqueous solutions were prepared in deionized water.

Assays. Protein was measured by the method of Lowry et al. (1951) with bovine plasma albumin as protein standard. Phosphorus was measured by using a modification (Rouser & Fleischer, 1967) of the method of Chen et al. (1956). D-β-Hydroxybutyrate dehydrogenase activity was measured spectrophotometrically as the rate of reduction of NAD^+ with β-hydroxybutyrate as substrate, as described previously (McIntyre et al., 1983). Counting of ^{14}C labels was performed with a Searle Mark III Model 6880 liquid scintillation counter using the ^{14}C-efficiency program with samples dissolved in aqueous counting scintillant from Amersham (Arlington Heights, IL).

Preparation of D-β-Hydroxybutyrate Dehydrogenase. D-β-Hydroxybutyrate apodehydrogenase, i.e., the inactive enzyme devoid of lipid, was purified essentially as described by

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MPL, the mixture of 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.

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Bock & Fleischer (1974, 1975) and stored in a liquid nitrogen refrigerator at 1–2 mg/mL in 0.4 M LiBr, 5 mM Hepes, and 5 mM dithiothreitol, pH 7.0. The specific activity of the enzyme preparations used in this study ranged between 125 and 150 μmol of NAD^+ reduced/(min·mg of protein) at 37 °C, after optimal activation with mitochondrial phospholipids (see below).

Preparation of Mitochondrial Phospholipid Vesicles. Mitochondrial phospholipids (MPL) (Fleischer et al., 1967) were isolated and microdispersed by dialysis from a solution in butanol–cholate vs. 20 mM Tris-HCl–1 mM EDTA, pH 8.1, to obtain small unilamellar vesicles (Fleischer & Fleischer, 1967). Prior to use for activation of the enzyme, the MPL vesicles were briefly sonicated (3–5 min) in a bath sonicator, centrifuged at 100000g for 60 min, and filtered through a 0.22-μm Triton-free filter (Millipore Corp., Bedford, MA).

Activation of D-β-Hydroxybutyrate Dehydrogenase with Phospholipid Vesicles and Removal of Dithiothreitol. Purified D-β-hydroxybutyrate apodehydrogenase was activated with MPL as follows. The enzyme (0.8 mg) was admixed with MPL vesicles (80 μg of lipid phosphorus) in a total volume of 4 mL of buffer containing 20 mM Tris-HCl, 10 mM DTT, and 1 mM EDTA, pH 8.1. After incubation for 1 h at 25 °C, the active enzyme–MPL complex was transferred under argon to a dialysis bag and dialyzed at 4 °C against 4 L of N_2-saturated buffer, containing 5 mM Hepes, 1 mM EDTA, pH 7.0, and either 50 mM LiBr or 50 mM NaCl. During dialysis, N_2 was bubbled through the buffer to exclude oxygen. The dialysis buffer was changed after 12 and 24 h. The enzyme–MPL complex, free of DTT, was collected after 36 h of dialysis. Specific enzymic activities after dialysis were found to be between 120 and 140 μmol of NAD^+ reduced/(min·mg of protein) at 37 °C. In the absence of DTT, the enzyme is susceptible to inactivation by air oxidation. Precautions taken to minimize oxidation included use of a nitrogen or argon atmosphere and inclusion of EDTA in the buffers. Dialysis tubing was treated as described previously (Latruffe et al., 1980).

Modification of D-β-Hydroxybutyrate Dehydrogenase with N-Ethylmaleimide. Derivatization with *N*-ethylmaleimide or *N*-[^{14}C]ethylmaleimide of the enzyme–MPL complex, free of DTT, was carried out in sealed vials under nitrogen at pH 7.0. At this pH, *N*-ethylmaleimide is specific for thiol groups (Riordan & Vallee, 1967). The enzyme was diluted to 0.14 mg/mL in buffer containing 5 mM Hepes, 1 mM EDTA, pH 7.0, and either 50 mM LiBr or 50 mM NaCl. Other experimental conditions, including the addition of substrates and/or nucleotides, are given in the figure legends and table footnotes. A stock solution of *N*-[^{14}C]ethylmaleimide (5 mM) in 5 mM Hepes (pH 7.0) was prepared by addition of the *N*-[^{14}C]-ethylmaleimide (provided as a solution in pentane) to the aqueous buffer in a sealed vial; after the solution was vortexed, the pentane was removed with a stream of nitrogen. In some experiments, the *N*-[^{14}C]ethylmaleimide in pentane was added directly to the reaction mixture and the pentane removed in like manner after vortexing. *N*-[^{14}C]Ethylmaleimide was added to the reaction mixtures to a 5-fold excess per enzyme monomer (or higher amounts as indicated). Aliquots (50 μL) were removed at appropriate time intervals and added to a small volume of DTT (final concentration 5 or 10 mM) to stop the reaction. The measured enzymic activity was correlated with ^{14}C incorporation from *N*-[^{14}C]ethylmaleimide using acid precipitation essentially as described previously (Latruffe et al., 1980). Radioactive *N*-ethylmaleimide was used as supplied without dilution by carrier. The amount of bound label was calculated by using the specific radioactivity given by the supplier and is expressed per monomer of D-β-hydroxybutyrate dehydrogenase (31 500 daltons; Bock & Fleischer, 1975). It should be noted that considerable care has to be taken to avoid scatter in the experimental data for determining inactivation stoichiometry values, especially at higher ratios of *N*-[^{14}C]-ethylmaleimide to enzyme. We found that the use of Ultra-Clear centrifuge tubes (Beckman Instruments, Spinco Division, Palo Alto, CA) for the acid precipitation steps introduced significantly more scatter as compared with using cellulose nitrate or propionate tubes from the same company.

NADH Binding Studies. Native and *N*-ethylmaleimide-modified D-β-hydroxybutyrate dehydrogenase–MPL complexes (75 μg of protein/mL) in N_2-saturated buffer (50 mM NaCl, 5 mM Hepes, 5 mM DTT, and 1 mM EDTA, pH 8.0) were used for NADH binding studies. Measurements were also made at different enzyme concentrations (40, 80, or 200 μg of protein/mL) with comparable results. Tryptophylalanine was used as a reference peptide. Three methods were used to characterize the NADH binding: (1) quenching of intrinsic protein fluorescence by NADH binding (λ_{ex} 290 nm, λ_{em} 340 nm); (2) resonance energy transfer from protein (tryptophan) to NADH (λ_{ex} 290 nm, λ_{em} 455 nm); (3) enhancement of NADH fluorescence on binding to the enzyme (λ_{ex} 340 nm, λ_{em} 455 nm). Excitation and emission spectra were recorded both prior to NADH addition and after addition of the maximum amount of NADH. There was no detectable change in the excitation or emission maxima for the derivatized enzyme as compared with those obtained with native enzyme. The fluorescence intensities (arbitrary units) were measured as a function of the NADH concentration by using a Perkin-Elmer MFP-44B fluorometer. Double-reciprocal plots of fluorescence intensity vs. NADH concentration were used to estimate K_d values (Latruffe et al., 1980). For estimation of K_d values for the enzyme–MPL complexes, the fluorescence values were corrected by subtraction of background signal due to lipid alone. For the tryptophylalanine reference peptide, the double-reciprocal plots intercepted the origin, indicating that the signals do not saturate (cf. Figure 3).

Results

D-β-Hydroxybutyrate dehydrogenase is inactivated by modification with *N*-ethylmaleimide. The rates of inactivation of the enzyme–MPL complex in the absence or in the presence of NADH or NAD^+ are shown in Figure 1. In the absence of coenzyme, D-β-hydroxybutyrate dehydrogenase is derivatized and inactivated by *N*-ethylmaleimide with an inactivation stoichiometry of one *N*-ethylmaleimide per enzyme monomer (Latruffe et al., 1980). The presence of 5 mM NADH is effective in protecting against inactivation by *N*-ethylmaleimide, whereas 5 mM NAD^+ only slightly reduces the rate of inactivation. Concomitant with inactivation, *N*-[^{14}C]-ethylmaleimide label is incorporated into the protein. In the presence of NAD^+ there is an approximate 50% decrease in incorporation of ^{14}C label as compared with incorporation in the absence of nucleotide. In Figure 2, the data have been replotted to correlate derivatization with loss of D-β-hydroxybutyrate dehydrogenase activity which shows a stoichiometry of inactivation of 0.5 in the presence of either 5 mM NAD^+ or 5 mM NADH.

The half-time of inactivation of D-β-hydroxybutyrate dehydrogenase by *N*-ethylmaleimide is affected by the addition of nucleotide, substrate, or the competitive inhibitor 2-methylmalonate (Table I). Substrates and nucleotides either separately or together alter the rate of inactivation of the enzyme although there are significant differences in the pro-

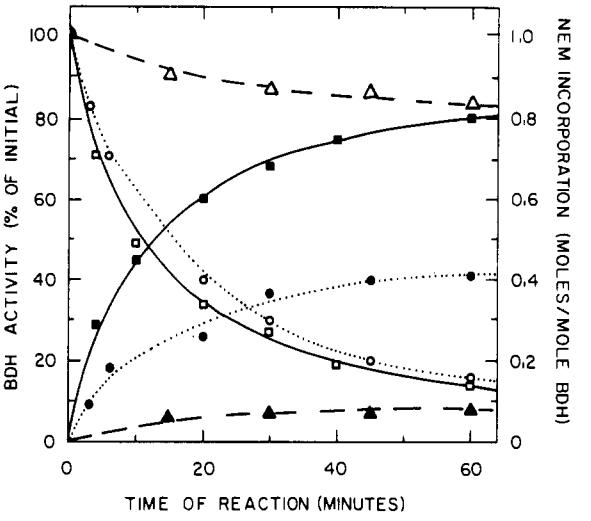

FIGURE 1: Time course of derivatization and inactivation of D-β-hydroxybutyrate dehydrogenase (BDH) by *N*-ethylmaleimide (NEM). The BDH–phospholipid complex was formed by incubation of the apodehydrogenase with mitochondrial phospholipid (100 μg of lipid phosphorus/mg of BDH) for 2 h at room temperature to obtain optimal activation [150 μmol of NAD^+ reduced/(min·mg of BDH) at 37 °C]. After dialysis vs. 50 mM LiBr, 5 mM Hepes, and 1 mM EDTA, pH 7.0, to remove DTT, the enzyme was diluted in the same buffer to a final concentration of 0.14 mg/mL (4.4 μM BDH subunit). *N*-[^{14}C]Ethylmaleimide was added to a final concentration of 22 μM (5 mol/mol of BDH subunit). Incubation was carried out at ice temperature either with no additions (□, ■) or in the presence of 5 mM NAD^+ (○, ●) or 5 mM NADH (△, ▲). At the times indicated, aliquots (50 μL) were removed, made 5 mM in DTT to stop the reaction, and assayed for BDH activity (open symbols) and incorporation of ^{14}C label (filled symbols). The initial specific activity prior to addition of NEM was 140 μmol of NAD^+ reduced/(min·mg of BDH) at 37 °C. There was no detectable loss of activity during 3 h of incubation in the absence of NEM.

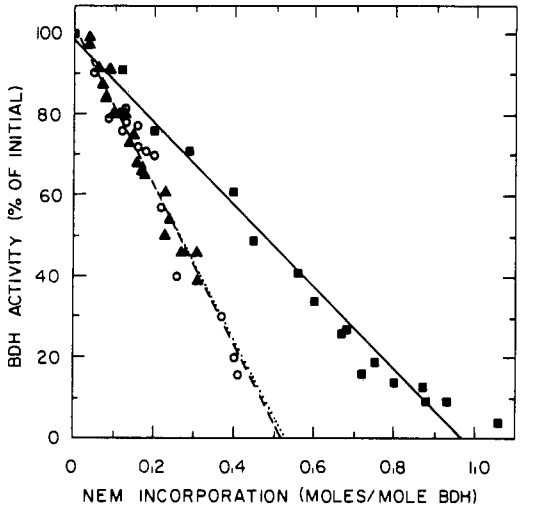

FIGURE 2: Stoichiometry of inactivation of D-β-hydroxybutyrate dehydrogenase (BDH) by *N*-ethylmaleimide (NEM). Data from several experiments similar to that shown in Figure 1 have been replotted to correlate loss of BDH activity with derivatization of BDH monomer by *N*-ethylmaleimide. The initial activity varied between 120 and 140 μmol of NAD^+ reduced/(min·mg). Incubation was carried out at 0 °C in 50 mM LiBr, 5 mM Hepes, and 1 mM EDTA (pH 7.0) either with no additions (■—■) or with 5 mM NAD^+ (○···○) or 5 mM NADH (▲– – –▲); the various points represent different times of incubation to a maximum of 2, 1.5, and 7 h, respectively.

tection afforded by each ligand. NADH offers appreciable protection in that the rate of inactivation is slowed down ~8-fold, whereas NAD^+ has little effect. The effect of substrate is not as marked as with NADH. Acetoacetate does not significantly change the rate of inactivation whereas β-hydroxybutyrate and 2-methylmalonate slow inactivation 3–4-fold. Although both NAD^+ and acetoacetate have little effect when added separately, together they increase the half-time of inactivation 4-fold.

As with NAD^+ or NADH, the addition of substrate either alone or together with nucleotide results in an inactivation stoichiometry of approximately 0.5 (Table I). The plot to obtain the inactivation stoichiometry for D-β-hydroxybutyrate dehydrogenase in the presence of acetoacetate was biphasic. Although inactivation to ~25% residual activity extrapolated to a stoichiometry of about 0.5, at longer incubation times, with <25% residual activity, a deviation from linearity extrapolating to an incorporation of approximately one ^{14}C label per enzyme monomer was observed. When the enzyme was protected with NAD^+ and 2-methylmalonate, inactivation was obtained only after prolonged incubation with a large (up to 100-fold) excess of *N*-ethylmaleimide (Table I). With only 5-fold excess of *N*-ethylmaleimide, essentially no activity was lost even after several hours of incubation although ~0.2 mol was incorporated per enzyme monomer. With 100-fold excess of reagent, 0.9 mol of ^{14}C label was incorporated rapidly (<5 min) without loss of enzymatic activity in the presence of NAD^+ and 2-methylmalonate. Activity was subsequently lost with a half-time of approximately 100 min with a stoichiometry of ~0.5. Under all conditions, prolonged incubation with 40–60 mol of *N*-ethylmaleimide/mol of enzyme monomer resulted in a total incorporation of ~2 mol of ^{14}C label/mol of enzyme subunit with essentially complete loss of enzymic activity.

In order to investigate the interaction of modified D-β-hydroxybutyrate dehydrogenase with NADH, two samples of enzyme were inactivated with *N*-ethylmaleimide, one without a protecting agent and the other in the presence of 5 mM NAD^+ during inactivation. This results in incorporation of 1.0 and 0.5 equiv, respectively (cf. Table I), with essentially complete loss of enzymic activity, in both cases, to a residual activity of less than 2%. After dialysis, NADH binding to these samples was compared with binding of NADH to the native enzyme–MPL complex. Three fluorescence approaches were used to characterize the interaction of NADH with enzyme, and the results of each are illustrated in Figure 3. Quenching of intrinsic fluorescence (Figure 3A) of the native enzyme is indicative of NADH binding. With the *N*-ethylmaleimide-modified enzyme samples, the quenching is smaller but significantly greater than that of the lipid control without enzyme. From double-reciprocal plots (inset, in Figure 3A) the apparent dissociation constants were ~20 and ~50 μM for native and modified enzyme, respectively. There is no significant difference in the quenching of intrinsic fluorescence curves between enzyme derivatized with *N*-ethylmaleimide in the presence and that in the absence of NAD^+ (i.e., labeled with either 0.5 or 1.0 equiv). It should be noted that the initial value of the intrinsic fluorescence of the modified enzyme was approximately half that of the native enzyme, suggesting an altered environment of the tryptophan(s) in the derivatized enzyme. The fluorescence of the control peptide, tryptophylalanine, was quenched upon addition of NADH but to a lower extent than either the native or modified enzyme although the initial value for fluorescence was similar to that of modified enzyme (Figure 3). Further, the double-reciprocal plot of quenching of intrinsic fluorescence of tryptophylalanine by NADH intercepts the origin (inset in Figure 3A); i.e., it is nonsaturating, indicating that, in this case, the quenching was not due to binding of the nucleotide (Cantor & Schimmel, 1980).

Table I: Derivatization of D-β-Hydroxybutyrate Dehydrogenase with *N*-Ethylmaleimide: Modulation of Inactivation Rate and Inactivation Stoichiometry by Nucleotides and Substrates[a]

additions[b]	time for 50% inactivation[c] (min)	inactivation stoichiometry[d] (mol of NEM/mol of BDH)	no. of expt
none	12 ± 2	1.0 ± 0.1	6
NAD^+	18 ± 2	0.5 ± 0.1	3
NAD^+ + acetoacetate	50 ± 5	0.5 ± 0.1	3
NAD^+ + 2-methylmalonate[e]	∞	0.5 ± 0.2	3
NADH	100 ± 10	0.5 ± 0.1	4
NADH + β-hydroxybutyrate	100 ± 10	0.5 ± 0.1	2
NADH + 2-methylmalonate[f]	200 ± 20	0.6 ± 0.2	3
acetoacetate[g]	13 ± 2	0.4 ± 0.1	4
β-hydroxybutyrate	37 ± 5	0.6 ± 0.1	4
2-methylmalonate	50 ± 5	0.6 ± 0.1	1

[a] Derivatization of D-β-hydroxybutyrate dehydrogenase (BDH) with *N*-ethylmaleimide (NEM) was carried out as described in Figure 1 by using the active BDH–mitochondrial phospholipid complex (100 μg of lipid phosphorus/mg of BDH) with addition of 5 mol of NEM/mol of BDH subunit. All values are given as the mean ± standard deviation of at least two series of measurements in each experiment. The initial specific activity of samples used in different experiments varied between 120 and 140 μmol of NAD^+ reduced/(min·mg of BDH). [b] The BDH–phospholipid complex was incubated at 0.14 mg/mL in 50 mM LiBr, 5 mM Hepes, and 1 mM EDTA, pH 7.0, with the indicated additions of nucleotides, substrates, or the competitive inhibitor 2-methylmalonate, at the following concentrations: NAD, 5 mM (K_i = 1.7 mM); NADH, 5 mM (K_i = 0.05 mM); acetoacetate, 10 mM (K_m = 1.6 mM); DL-β-hydroxybutyrate, 20 mM (K_m for D isomer = 2.0 mM); 2-methylmalonate, 10 mM (K_i = 0.1 mM; Tan et al., 1975). The K_i and K_m values for nucleotides and substrates are from Nielsen et al. (1973). BDH has an ordered sequential reaction mechanism (Nielsen et al., 1973) so that the K_i is equivalent to the dissociation constant. [c] The values for the time to obtain 50% inactivation are given for derivatization at 0 °C. The rates were approximately twice as fast at 25 °C. The rates are reported as the time to obtain 50% inactivation since, with the amount of NEM used in these studies (5 mol/mol of BDH subunit), the rate of inactivation is not first order. At this low ratio of NEM to BDH, the concentration of NEM decreases significantly during the reaction especially since the reagent is susceptible to hydrolysis. Pseudo-first-order reaction rates, obtained at high NEM concentrations, are too rapid to be accurately measured. The time to obtain 50% inactivation with the low amount of NEM used in these studies provides a semiquantitative comparison under different conditions. [d] The stoichiometry of inactivation was determined from the decrease in activity and derivatization as a function of time by the graphic method shown in Figure 2. The total reaction time varied with the different samples depending on the rate of inactivation and ranged between 1.5 (in the presence of 5 mM NAD^+) and 10 h (in the presence of NADH plus 2-methylmalonate) (cf. Figure 2). For reaction conditions which give slow inactivation (i.e., in the presence of NADH), data were obtained for samples that were inactivated to less than 20% of the original activity; for the other conditions of more rapid inactivation, data were obtained for samples exhibiting 2% or less residual activity. The stoichiometry values were unaffected by the initial specific activity (120–140) of BDH. Derivatization was carried out at either 0 or 25 °C without any significant difference in the inactivation stoichiometry. Under all incubation conditions, subsequent addition of excess NEM (40–60 mol/mol of BDH) resulted in incorporation of ~2 mol of NEM/mol of BDH after overnight reaction. [e] In the presence of NAD^+ and 2-methylmalonate, there was no loss of activity during 3 h of incubation with 2 mol of NEM added per mol of BDH subunit, although 0.15 mol of NEM/mol of BDH was incorporated. Increasing the molar ratio of NEM to BDH resulted in increased incorporation without loss of activity followed by inactivation with a stoichiometry of 0.5 (average of data obtained at 20 and 100 mol of NEM/BDH monomer). At 100 mol of NEM/mol of BDH, approximately 0.9 mol of NEM was incorporated within 10 min after initiation of the reaction without significant loss of activity; subsequent loss in BDH activity, concomitant with incorporation of ^{14}C label, was observed with a time for 50% inactivation of ~100 min. The rapid initial labeling obtained under these conditions provides a method to selectively incorporate probes into the protein without loss of function. [f] In the presence of NADH and 2-methylmalonate, the plot of BDH activity vs. incorporation of NEM, to obtain the inactivation stoichiometry, exhibited significantly more scatter than other data probably due to the extended reaction times required. [g] The plot to obtain the inactivation stoichiometry in the presence of acetoacetate was biphasic, with ~75% inactivated with stoichiometry extrapolating to 0.4. At longer incubation times (>30 min) incorporation approximated 1.0 mol of NEM/BDH monomer.

Resonance energy transfer from protein to bound NADH is observed for the native enzyme (Figure 3B). For the modified enzyme, derivatized with *N*-ethylmaleimide either in the presence or in the absence of NAD^+ (i.e., either 0.5 or 1.0 equiv), there is no resonance energy transfer from protein to bound NADH, since the fluorescence yield is similar to that in the presence of lipid alone. With the control peptide tryptophylalanine, there is some resonance energy transfer to NADH which likely reflects a nonspecific interaction also observed in the quenching of intrinsic fluorescence (Figure 3A). The fluorescence of NADH is enhanced upon binding to the active D-β-hydroxybutyrate dehydrogenase–MPL complex as compared with the fluorescence obtained with a lipid control sample without enzyme (Gazzotti et al., 1974). For the enzyme inactivated with *N*-ethylmaleimide in the presence or absence of NAD^+, the fluorescence of NADH is comparable to that with lipid alone, i.e., it is not enhanced (Figure 3C).

Discussion

These studies show that *N*-ethylmaleimide modification of D-β-hydroxybutyrate dehydrogenase, in the presence of coenzyme or substrate, results in loss of enzymic activity with a stoichiometry of inactivation of 0.5 equiv incorporated per enzyme monomer. The reduction in inactivation stoichiometry from 1.0 to 0.5 by the addition of ligands to the enzyme indicates that substrate and/or coenzyme protects a nonessential sulfhydryl from being derivatized when limiting amounts of *N*-ethylmaleimide (5 mol of enzyme subunit) are added. Further, the rate of inactivation of the enzyme is significantly slower in the presence of nucleotide or substrate so that these ligands also afford some protection of the essential sulfhydryl from reaction with *N*-ethylmaleimide. The protection by NADH is more pronounced than that by NAD^+ which may reflect the weaker binding of the oxidized coenzyme (cf. Table I) or be due to a slightly different orientation of NAD^+ in the nucleotide binding site as compared with NADH. Substrate or substrate analogues, either alone or together with coenzyme to form ternary complexes,[2] also modulate the rate of inactivation of D-β-hydroxybutyrate dehydrogenase by *N*-ethylmaleimide. The different rates of inactivation in the presence of the different ligands likely reflect both differences in the binding constants and also proximity of the ligand to the essential sulfhydryl. An inactivation

[2] The term abortive ternary complex refers to a complex of the active enzyme–phospholipid complex together with nucleotide and substrate that are both either oxidized (e.g., NAD^+ and acetoacetate) or reduced (e.g., NADH and β-hydroxybutyrate). For D-β-hydroxybutyrate dehydrogenase such ternary complexes are in a sense quaternary since the active form of the enzyme is already a binary complex of protein and phospholipid prior to addition of substrate and nucleotide. Ternary complex is used here for consistency with the data of Fritzsche and co-workers for other dehydrogenases (Fritzsche et al., 1984).

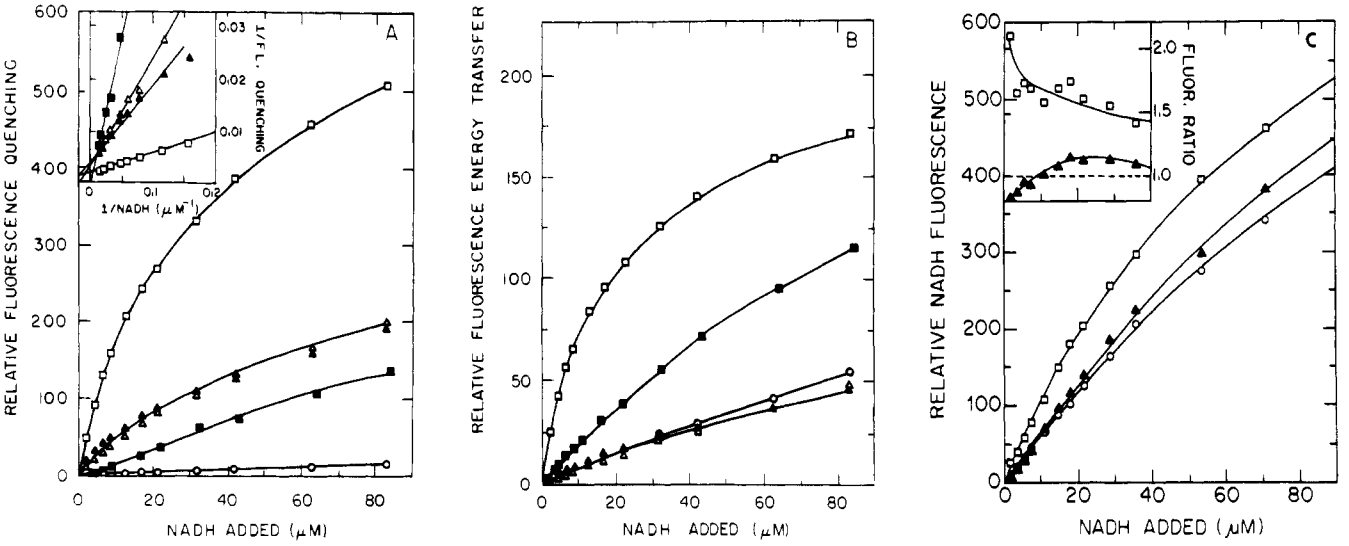

FIGURE 3: Binding of NADH to D-β-hydroxybutyrate dehydrogenase (BDH) before and after derivatization with *N*-ethylmaleimide. The active BDH–MPL complex was prepared as described in Figure 1, dialyzed to remove DTT and inactivated by treatment with *N*-ethylmaleimide (5 mol/mol of BDH subunit) overnight at 0 °C to <2% of the initial activity. The samples (1.5 mL each) were then dialyzed against 3 × 1000 volumes of 50 mM NaCl, 5 mM Hepes, 5 mM DTT, and 1 mM EDTA, pH 8.0. The protein was diluted to 75 μg of BDH/mL (2.4 μM) prior to addition of NADH. Similar results were obtained at 40, 80, or 200 μg of BDH/mL (not shown). Data are shown for underivatized enzyme (□), enzyme inactivated by *N*-ethylmaleimide (added at 5 mol/mol of BDH) either in the presence (▲) or in the absence (△) of 5 mM NAD^+, tryptophylalanine reference peptide (436 nM) (■), and MPL (○) at the same concentration as in the BDH–MPL complex (7.5 μg of lipid phosphorus/mL); 1 and 0.5 equiv of sulfhydryl are derivatized per BDH monomer (△, ▲), respectively. Each sample was titrated with NADH, and three fluorescence parameters were measured (arbitrary units) at each concentration of NADH. (A) Quenching of the intrinsic protein fluorescence (λ_{ex} 290 nm, λ_{em} 340 nm) shown as the decrease in fluorescence of the initial samples which were 790, 363, 365, 283, and 35 arbitrary units for (□), (▲), (△), (■), and (○), respectively. The inset is a double-reciprocal plot of the data which demonstrates the saturation behavior for the BDH samples and nonsaturating character for tryptophylalanine; i.e., the double-reciprocal plot intercepts the origin. The apparent dissociation constant (K_d) for NADH binding to BDH is determined from the quotient of the slope/intercept of the *y* axis (Latruffe et al., 1980). K_d values for underivatized enzyme (□) and enzyme inactivated by *N*-ethylmaleimide (▲, △) are ~20 μM and ~50 μM, respectively. (B) Fluorescence energy transfer from enzyme to bound NADH (λ_{ex} 290 nm, λ_{em} 455 nm). (C) Enhancement of NADH fluorescence (λ_{ex} 340 nm, λ_{em} 455 nm). These data were obtained at 200 μg of BDH/mL. For the *N*-ethylmaleimide-inactivated enzyme, data are shown only for the enzyme derivatized in the presence of NAD^+ (▲), i.e., 0.5 equiv of sulfhydryl derivatized. Similar data were obtained for the enzyme inactivated in the absence of NAD^+, i.e., 1.0 equiv derivatized (not shown). The inset at the upper left is a replot of the same data but as the relative fluorescence ratio which is calculated as the ratio of the fluorescence of NADH with BDH–MPL to that with MPL alone. For all three fluorescence measurements (quenching of protein fluorescence, fluorescence energy transfer, and enhancement of NADH fluorescence), the excitation and emission maxima were similar for native BDH and BDH derivatized and inactivated with *N*-ethylmaleimide (not shown).

stoichiometry of 0.5 is obtained in the presence of nucleotide or substrate, either separately or together, under all conditions tested. We conclude from this that D-β-hydroxybutyrate exhibits half-site reactivity; i.e., derivatization of one sulfhydryl group results in loss of activity of two polypeptide chains. The functional form of the enzyme reconstituted into phospholipid vesicles is a tetramer (McIntyre et al., 1983) although the enzyme activated with soluble lecithin shows a dimeric functional unit (McIntyre et al., 1978). The results reported here suggest that the tetramer consists of two functional units each consisting of two identical polypeptide chains. Modification of a single sulfhydryl results in loss of activity of one dimeric unit of the tetramer.

Thiol reagents have previously been reported to inactivate D-β-hydroxybutyrate dehydrogenase in submitochondrial vesicles from both rat liver (Latruffe & Gaudemer, 1974) and beef heart (Phelps & Hatefi, 1981a). We observe protection of the essential sulfhydryl of D-β-hydroxybutyrate dehydrogenase by nucleotide or substrate that differs somewhat from these previous reports. The differences likely reflect experimental variations, and since the previous studies were carried out for the enzyme in situ, the inactivation stoichiometry could not be measured directly. The power of using the purified enzyme is that the reaction stoichiometry has been determined both in the absence and in the presence of either substrate or coenzyme. With the purified enzyme, we have previously shown that D-β-hydroxybutyrate dehydrogenase has two sulfhydryl groups per polypeptide chain that are accessible to labeling with *N*-ethylmaleimide (Latruffe et al., 1980). We have recently confirmed this result and shown that the four remaining cysteines form disulfide bridges since they are not accessible even in the denatured enzyme (Fleer et al., 1984). In the study reported here, a maximal incorporation of ~2 mol of *N*-[^{14}C]ethylmaleimide/mol of enzyme subunit is observed. As reported previously (Latruffe et al., 1980), we confirm that only one of these is essential for function. The new aspect of this study is that we can obtain half-site reactivity of the enzyme in the presence of substrate and/or coenzyme. The change in inactivation stoichiometry from 1 to 0.5 by the addition of nucleotide or substrate could arise from a change in subunit association. Such an effect of NAD^+ on the association of subunits of glyceraldehyde-phosphate dehydrogenase has been reported (Smith & Schachman, 1971). An alternative explanation is that, in the absence of ligands, the essential and nonessential sulfhydryls are derivatized to about the same extent, whereas in the presence of the ligands, the labeling of the nonessential sulfhydryl is blocked (at the low ratio of *N*-ethylmaleimide to protein used in these studies). The result is incorporation of label only in the essential sulfhydryl moiety.

The addition of 2-methylmalonate to D-β-hydroxybutyrate dehydrogenase in the presence of coenzyme results in a significant increase in protection of the essential sulfhydryl compared with coenzyme alone (Table I). Methylmalonate is a competitive inhibitor for β-hydroxybutyrate (K_i = 0.1 mM) and uncompetitive with respect to acetoacetate (Tan et al., 1975). When 2-methylmalonate is added together with NAD^+, incorporation of label from *N*-ethylmaleimide (at

5-fold excess) occurred slowly without appreciable loss in activity, similar to results reported in our earlier study (Latruffe et al., 1980). We now show that, by increasing the *N*-ethylmaleimide to enzyme ratio by 20-fold, ~0.9 equiv of ^{14}C label is incorporated without appreciable loss in activity and then a slow inactivation with a stoichiometry of 0.5 is observed. The initial rapid derivatization without loss of activity provides the basis for a method to obtain a functional enzyme selectively derivatized with spin-label probes so as to characterize the anisotropic motion of the protein (McIntyre et al., 1984).

Although the reaction conditions are optimized to give specific reaction of the *N*-ethylmaleimide with sulfhydryl groups (Riordan & Vallee, 1967), it is possible that the very slow inactivation observed in the presence of coenzyme and 2-methylmalonate is occurring at a group other than a sulfhydryl. In this regard, a slow inactivation of the enzyme by *N*-ethylmaleimide is also observed when the essential sulfhydryl is protected by diamide treatment (Fleer et al., 1984). Whether the inactivation observed in the presence of 2-methylmalonate together with either NAD^+ or NADH is due to labeling of a sulfhydryl or another group, it is clear that the ligands afford significant protection of the essential sulfhydryl that reacts rapidly with *N*-ethylmaleimide in the absence of ligands or in the presnce of NAD^+ or substrate alone.

The interaction of NADH with D-β-hydroxybutyrate dehydrogenase has been studied by using three fluorescence characteristics that give separate insights into the effect of derivatization of the enzyme with *N*-ethylmaleimide on NADH binding. Quenching of intrinsic protein fluorescence shows that NADH binds to the *N*-ethylmaleimide-derivatized enzyme (labeled with either 0.5 or 1.0 equiv) albeit with a 2–3-fold higher apparent dissociation constant (Figure 3A). The amplitude of the intrinsic fluorescence of modified enzyme is about half that of native enzyme, suggesting that the *N*-ethylmaleimide-derivatized sulfhydryl is close to a tryptophan moiety and modifies its fluorescent properties. Resonance energy transfer from protein to bound NADH is lost subsequent to derivatization of the enzyme either in the absence of NAD^+ [as reported previously (Latruffe at al., 1980)] or in the presence of NAD^+ (Figure 3B). The loss of resonance energy transfer is likely due to a change in the orientation of the nucleotide with respect to the tryptophan involved in fluorescence transfer since such energy transfer is sensitive to the geometric arrangement of the two chromophoric moieties (Stryer, 1978). Further, the fluorescence of NADH bound to the derivatized enzyme is not enhanced (Figure 3C) whereas NADH fluorescence enhancement (fluorescence ratio in the presence vs. in the absence of enzyme) is observed for the native enzyme (Gazzotti et al., 1974). Thus, D-β-hydroxybutyrate dehydrogenase inactivated by *N*-ethylmaleimide (0.5 or 1.0 equiv) retains the ability to bind NADH. However, derivatization alters the nucleotide binding domain in that the intrinsic protein fluorescence is decreased and both resonance energy transfer from protein to bound NADH and enhancement of fluorescence of bound NADH are abolished.

Half-of-the-site reactivity has been documented for a number of multimeric enzymes (Huang et al., 1982). In succinyl-CoA synthetase of *Escherichia coli*, Moffet et al. (1972) showed that only one of two identical subunits could be phosphorylated at any one time. This enzyme was later shown to exhibit alternating site cooperativity (Bild et al., 1980). The half-site reactivity of alkaline phosphatase, in terms of formation of one phosphoryl enzyme per dimer, has been investigated by NMR. These studies indicated that there was substrate-induced cofactor migration such that the second subunit becomes incompetent in substrate binding, in accord with the observed negative cooperativity of binding reported for this enzyme (Otvos & Armitage, 1980; Coleman & Chlebowski, 1979). In chloroplast ATPase, complete inactivation of the multimer was obtained with modification of a single tyrosyl residue per F_1 complex (Cantley & Hammes, 1975). Similar half-of-site reactivity data for mitochondrial ATPase together with data for oxygen exchange between phosphate, ATP, and H_2O form the basis of the alternating site model for catalysis for the ATP synthetase (F_0–F_1 complex) (Boyer, 1979). Half-site reactivity has also been reported for dehydrogenases. For glyceraldehyde-3-phosphate dehydrogenase, the essential sulfhydryl is selectively inactivated with either half-site reactivity (2 equiv/tetramer) or full-site reactivity (4 equiv/tetramer) depending on the acylating reagent used (Seydoux et al., 1973). With isocitrate dehydrogenase, which is composed of three distinct types of subunits, nucleotide binding exhibits half-of-the-site reactivity (Ehrlich & Colman, 1982), whereas the inactivation stoichiometry by at least three protein derivatizing reagents is approximately one (King & Colman, 1983). Protein derivatization studies that result in multiple site labeling yield little information about active site structure. In a few cases, such multiple site labeling has proved useful in studies to characterize the effect of subunit–subunit interaction on function by reconstituting multimers consisting of both underivatized and inactivated subunits (Smith & Schachman, 1971). Such an approach to further characterize the nature of subunit–subunit interaction in D-β-hydroxybutyrate dehydrogenase after derivatization of only a single site would be complicated by the presence of the phospholipid membrane. Studies such as those cited above and also reported here in which unique-site derivatization is obtained reveal detailed information about the functional significance of the amino acid being modified. The unique aspect of the results reported here for D-β-hydroxybutyrate dehydrogenase is that single-site reactivity is obtained in the absence of ligands. Addition of nucleotide and/or substrate to the enzyme results in half-of-the-site reactivity of the essential sulfhydryl. Further, half-of-the-site reactivity has previously been observed only for enzymes which exhibit subunit cooperation; for D-β-hydroxybutyrate dehydrogenase there is, as yet, no evidence for cooperativity in the enzymic mechanism (Nielsen et al., 1973; Latruffe & Gaudemer, 1974) or nucleotide binding (Gazzotti et al., 1974; Latruffe et al., 1980).

Registry No. BDH, 9028-38-0; NAD, 53-84-9; NADH, 58-68-4; *N*-ethylmaleimide, 128-53-0; acetoacetic acid, 541-50-4; 2-methylmalonic acid, 516-05-2; β-hydroxybutyric acid, 300-85-6.

References

Bild, G. S., Janson, C. A., & Boyer, P. D. (1980) *J. Biol. Chem. 255*, 8109–8115.

Bock, H.-G., & Fleischer, S. (1974) *Methods Enzymol. 31*, 374–391.

Bock, H.-G., & Fleischer, S. (1975) *J. Biol. Chem. 250*, 5774–5781.

Boyer, P. D. (1979) in *Membrane Bioenergetics* (Lee, C. P., Schatz, G., & Ernster, L., Eds.) pp 461–479, Addison-Wesley, Reading, PA.

Brenner, S. C., McIntyre, J. O., Latruffe, N., Fleischer, S., Tuhy, P., Elion, J., & Mann, K. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 577.

Cantley, L. C., Jr., & Hammes, G. G. (1975) *Biochemistry 14*, 2976–2981.

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry Part II: Techniques for the Study of Biological Structure and Function*, pp 433–465, W. H. Freeman, San Francisco, CA.

Chen, P. S., Toribara, T., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Coleman, J. E., & Chlebowski, J. F. (1979) *Adv. Inorg. Biochem. 1*, 1–66.

Ehrlich, R. S., & Colman, R. F. (1982) *J. Biol. Chem. 257*, 4769–4774.

Fleer, E. A. M., & Fleischer, S. (1983) *Biochim. Biophys. Acta 749*, 1–8.

Fleer, E. A. M., McIntyre, J. O., & Fleischer, S. (1984) *Biochemistry* (following paper in this issue).

Fleischer, S., & Fleischer, B. (1967) *Methods Enzymol. 10*, 406–433.

Fleischer, S., Rouser, G., Fleischer, B., Casu, A., & Kritchevsky, G. (1967) *J. Lipid Res. 8*, 170–180.

Fleischer, S., Bock, H.-G. O., & Gazzotti, P. (1974) in *Membrane Proteins in Transport and Phosphorylation* (Klingenberg, M., & Azzone, G. F., Eds.) pp 125–136, North-Holland Publishing Co., Amsterdam.

Fleischer, S., McIntyre, J. O., Churchill, P., Fleer, E., & Maurer, A. (1983) in *Structure and Function of Membrane Protins* (Quagliarello, E., & Palmieri, F., Eds.) pp 283–300, Elsevier, Amsterdam, The Netherlands.

Fritzsche, T. M., McIntyre, J. O., Trommer, W. E., & Fleischer, S. (1984) *Biochim. Biophys. Acta* (in press).

Gazzotti, P., Bock, H.-G. O., & Fleischer, S. (1974) *Biochem. Biophys. Res. Commun. 58*, 309–315.

Huang, C. Y., Rhee, S. G., & Chock, P. B. (1982) *Annu. Rev. Biochem. 51*, 935–971.

King, M. M., & Colman, R. F. (1983) *Biochemistry 22*, 1656–1665.

Latruffe, N., & Gaudemer, Y. (1974) *Biochimie 56*, 435–444.

Latruffe, N., Brenner, S. C., & Fleischer, S. (1980) *Biochemistry 19*, 5285–5290.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

McIntyre, J. O., Holladay, L. A., Smigel, M., Puett, D., & Fleischer, S. (1978) *Biochemistry 17*, 4169–4177.

McIntyre, J. O., Fleer, E. A. M., & Fleischer, S. (1982) *Eur. Bioenerg. Conf. 2nd*, 509–510.

McIntyre, J. O., Churchill, P., Maurer, A., Berenski, C. J., Jung, C. Y., & Fleischer, S. (1983) *J. Biol. Chem. 258*, 953–959.

McIntyre, J. O., Robinson, B. H., & Fleischer, S. (1984) *Biophys. J. 45*, 238.

Moffet, F. J., Wang, T., & Bridger, W. A. (1972) *J. Biol. Chem. 247*, 8139–8144.

Nielsen, N. C., Zahler, W. L., & Fleischer, S. (1973) *J. Biol. Chem. 248*, 2556–2562.

Otvos, J. D., & Armitage, I. M. (1980) *Biochemistry 19*, 4031–4043.

Phelps, D. C., & Hatefi, Y. (1981a) *Biochemistry 20*, 453–458.

Phelps, D. C., & Hatefi, Y. (1981b) *Biochemistry 20*, 459–463.

Riordan, J. F., & Vallee, B. L. (1967) *Methods Enzymol. 11*, 541–548.

Rouser, G., & Fleischer, S. (1967) *Methods Enzymol. 10*, 385–406.

Seydoux, F., Berhard, S., Pfenninger, O., Payne, M., & Malhotra, O. P. (1973) *Biochemistry 12*, 4290–4300.

Smith, G. D., & Schachman, H. K. (1971) *Biochemistry 10*, 4576–4588.

Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.

Tan, A. W. H., Smith, C. M., Aogaichi, T., & Plaut, G. H. E. (1975) *Arch. Biochem. Biophys. 166*, 164–173.