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Essential Sulfhydryl for Reduced Nicotinamide Adenine Dinucleotide Binding in D-β-Hydroxybutyrate Dehydrogenase†

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ABSTRACT: Chemical derivatization studies have been directed at the sulfhydryl group of D-β-hydroxybutyrate dehydrogenase, a lipid-requiring enzyme. Reaction with *N*-ethylmaleimide leads to progressive and parallel loss of both enzymic activity and coenzyme binding. Both functions are lost when 1 equiv of sulfhydryl is derivatized per mol of enzyme. Inactivation of the enzyme with methylmercury or with air oxidation also leads to loss of coenzyme binding. We conclude that a single "essential" sulfhydryl is required for coenzyme binding and consequently for enzymic activity. Only two "accessible" cysteine residues can be derivatized even at high levels of *N*-ethylmaleimide, whereas derivatization of the remaining three "inacessible" cysteines requires denaturation of the enzyme. The enzyme can apparently be labeled in the accessible, but nonessential, sulfhydryl in the presence of coenzyme which protects against inactivation by *N*-ethylmaleimide. Such selective covalent labeling of the nonessential sulfhydryl makes possible future biophysical studies of enzyme–phospholipid interaction of a functional enzyme using extrinsic probes.

D-β-Hydroxybutyrate dehydrogenase is a lipid-requiring enzyme with an absolute requirement of lecithin for function. The apodehydrogenase, which is devoid of lipid, has been purified to homogeneity. It is inactive, but can be made functional by forming an active enzyme–phospholipid complex. For a review, see Fleischer et al. (1974).

D-β-Hydroxybutyrate dehydrogenase is perhaps the most extensively studied lipid-requiring enzyme, and we are studying it to understand the molecular basis of the role of lipid (Gazzotti et al., 1974) and the nature of lipid–protein interaction (Fleischer et al., 1979). In this regard, the chemistry of this enzyme is now being addressed, including primary sequence and chemical derivatization studies in collaboration with Dr. Kenneth Mann of the Mayo Foundation. In the present study, we demonstrate the importance of a single sulfhydryl moiety for both enzymic function and coenzyme (NADH)[1] binding. A preliminary report of this work has appeared (Latruffe et al., 1979).

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[1] Abbreviations used: BAL, British Anti-Lewisite (2,3-mercapto-1-propanol); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MalNEt, *N*-ethylmaleimide; Mops, 4-morpholinepropanesulfonic acid; MPL, mitochondrial phospholipids; NAD(H), nicotinamide adenine dinucleotide (reduced); Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EPR, electron paramagnetic resonance.

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Experimental Procedures

Chromatopure grade NADH from P-L Biochemicals was used for binding studies. Lithium bromide from Matheson Coleman and Bell was prepared as a 4 M stock solution which was purified by filtration sequentially through a paper prefilter, an activated carbon column, and a 0.22-μm Millipore filter. [^{14}C]MalNEt (*N*-[1,2-^{14}C]ethylmaleimide), with a specific radioactivity of 4.1 mCi/mmol, was obtained from RPI and was stored in pentane under nitrogen at −20 °C. Immediately before each use, the pentane was evaporated under a stream of nitrogen, and the MalNEt was dissolved in aqueous buffer of 5 mM Hepes, pH 7.0.

Protein concentrations were determined by using bovine serum albumin as standard (Lowry et al., 1951). Samples containing DTT were assayed for protein after carboxymethylation with iodoacetate (Ross & Schatz, 1973). The protein concentration of the purified D-β-hydroxybutyrate dehydrogenase was also checked by absorbance [$A_{280,0.1\%,1cm}$ = 1.0; H.-G. O. Bock and S. Fleischer (unpublished results)].

Bovine heart mitochondria were prepared by modification (Bock & Fleischer, 1974) of the procedure described by Blair (1967). D-β-Hydroxybutyrate dehydrogenase was purified from bovine heart mitochondria according to Bock & Fleischer (1974, 1975) and was stored in a liquid nitrogen refrigerator in 5 mM Hepes, 5 mM DTT, and 0.4 M LiBr, pH 7.0.

Mitochondrial phospholipid (MPL) and its purified phospholipid components (phosphatidylcholine, phosphatidylethanolamine, and diphosphatidylglycerol) were prepared from bovine heart mitochondria as described previously (Gazzotti et al., 1975). Mitochondrial phospholipid contains the mixture of phospholipids in beef heart mitochondria, the neutral lipids having been removed by silicic acid chromatography (Fleischer et al., 1967). Prior to use for reactivation of the enzyme, the phospholipids were dispersed either by sonication or dialysis from cholate solution (Fleischer & Klouwen, 1961), under nitrogen, in 20 mM Tris-HCl and 1 mM EDTA, pH 8.1, at room temperature as previously described (Gazzotti et al., 1975). Phosphorus was measured by using a modification (Rouser & Fleischer, 1967) of the procedure of Chen et al. (1956).

Enzymic activity was assayed by measuring the rate of NADH production at 340 nm using a Gilford Model 2000 spectrophotometer in the standard medium described previously [pH 7.35, 37 °C; Bock & Fleischer (1975)]. Enzyme–phospholipid complexes were formed by preincubation for 15 min at room temperature unless stated otherwise (Isaacson et al., 1979). For assay, 5–10 μg of apodehydrogenase was activated with optimal amounts of mitochondrial phospholipid (60–80 μg of lipid phosphorus/mg of apoenzyme) in a 50-μL final volume containing 20 mM Tris-HCl, 1 mM EDTA, and 5 mM DTT, pH 8.1. Activity was measured by the addition of a small aliquot (10–20 μL) of the complex to a cuvette containing the assay mixture preequilibrated at 37 °C. One unit of specific activity is equivalent to the reduction of 1 μmol of NAD (min mg of enzyme)$^{-1}$.

Prior to derivatization with MalNEt, the solution containing apodehydrogenase was dialyzed at 4 °C under nitrogen to remove DTT. The dialysis buffer contained 5 mM Hepes, 0.4 M LiBr, 1 mM ascorbate, and 2 mM EDTA, pH 7.0. In the absence of DTT, the apodehydrogenase is readily inactivated by air oxidation. Precautions taken to minimize oxidation included use of a nitrogen atmosphere and inclusion of EDTA and ascorbate in the buffers. Dialysis tubing was treated sequentially with 1% acetic acid, 1% sodium carbonate, 1 mM EDTA, and finally 0.1 M ascorbate (Brewer et al., 1974). The apodehydrogenase retained >90% of its initial activation capacity after dialysis for 16–20 h under these conditions.

Derivatization of the apodehydrogenase or the enzyme–phospholipid complex with [^{14}C]MalNEt was carried out in sealed vials under nitrogen at pH 7.0. At this pH, MalNEt is specific for thiol groups (Riordan & Vallee, 1967). Prior to use, all solutions were degassed and saturated with nitrogen. Other experimental conditions are given in the figure and table legends. Derivatization was stopped at the indicated time by transferring an aliquot of the reaction mixture to a small centrifuge tube at 0 °C containing a 500-fold molar excess of 2-mercaptoethanol or DTT. After removing aliquots from the tube for measurement of enzymic activity, the remainder was assayed for bound radioactivity. To achieve quantitative recovery of labeled enzyme, we added bovine serum albumin as carrier prior to precipitation with 7% perchloric acid at 0 °C. Under the conditions used, carrier albumin did not bind any label. Following acid precipitation, the mixture was centrifuged at 160000*g* for 2 min in a Beckman Airfuge centrifuge. The supernatant was removed by aspiration and the precipitate was dissolved in 100 μL of 0.1 M NaOH at room temperature. The precipitation, centrifugation, and dissolution were repeated once and the sample was transferred to a scintillation vial for counting. In samples containing dodecyl sulfate, acid precipitation was incomplete. For these samples, the reaction was quenched with DTT, and unbound label was removed by exhaustive dialysis at room temperature against 0.5% dodecyl sulfate and 5 mM Hepes, pH 7.0. Radioactivity was determined in 10 mL of ACS scintillation cocktail (Amersham) by using a Mark III Searle Analytic 81 liquid scintillation counter with DPM accessory. Radiactive MalNEt was used as supplied without dilution by carrier, and the amount of bound label was calculated by using the specific radioactivity provided by the supplier. Incorporation of label into D-β-hydroxybutyrate hydrogenase is expressed per single chain of 31 500 daltons (Bock & Fleischer, 1975).

Results

Inactivation and Labeling with N-Ethylmaleimide. *N*-Ethylmaleimide was used to study the role of sulfhydryl groups in the function of D-β-hydroxybutyrate dehydrogenase. The derivatization reactions were carried out at pH 7.0, at which MalNEt is specific for sulfhydryl groups (Riordan & Vallee, 1967). As confirmation that the reaction occurred specifically at sulfhydryl groups, the enzyme was treated with mersalyl, a thiol-specific mercurial. The reaction of mersalyl is readily reversed by excess thiols, regenerating the original sulfhydryl group. Mersalyl was found to protect the enzyme from derivatization with MalNEt. The mersalyl-treated enzyme, after incubation with MalNEt under conditions which readily inactivate the untreated enzyme, was restored to full activity when assayed for enzymic activity in the presence of excess DTT. Thus, the site of inactivation by MalNEt appears to be a sulfhydryl group.

Labeling of the apodehydrogenase by using a molar ratio of MalNEt to enzyme of 7.7-fold leads to derivatization of the enzyme with a corresponding loss of enzymic activity (Figure 1A). Enzymic activity of the apodehydrogenase, which requires phospholipid for function, is expressed after reactivation with mitochondrial phospholipids. The enzyme–phospholipid complex is labeled and inactivated more slowly than the apodehydrogenase using the same conditions. Despite the different rates, the stoichiometry of inactivation is the same for both apodehydrogenase and the active enzyme–phospholipid complex. Figure 1B shows the complete loss of enzymic

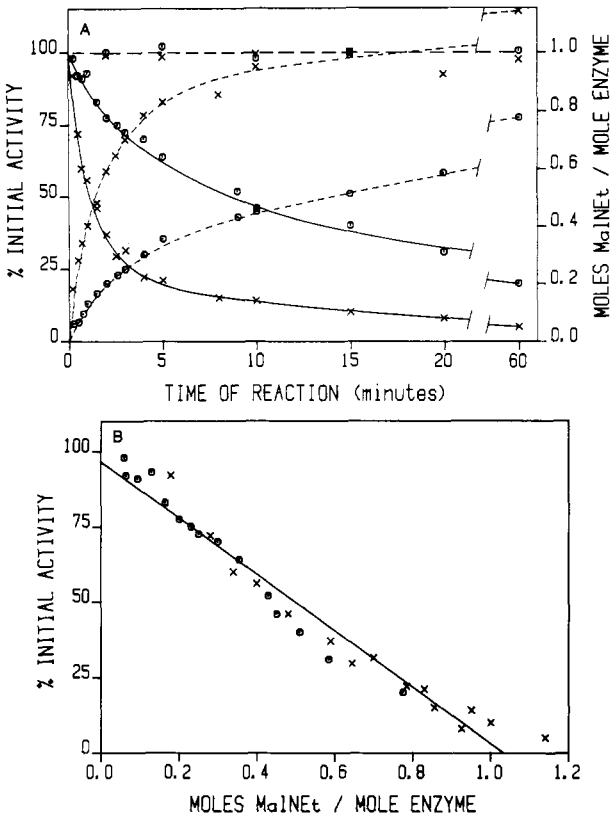

FIGURE 1: Derivatization of D-β-hydroxybutyrate dehydrogenase by *N*-ethylmaleimide. (A) Time course of inactivation and labeling. Apoenzyme (×) or enzyme–phospholipid complex (⊙) was labeled with MalNEt at 0 °C as described under Experimental Procedures. Incorporation of label (---), loss of activity (—), and enzymic activity in the absence of MalNEt (--) are shown. The enzyme–phospholipid complex was preformed with mitochondrial phospholipid for 15 min at room temperature with 120 μg of lipid phosphorus/mg of enzyme. The reaction mixture contained 82 μg of BDH/mL (2.5 μM) in 5 mM Hepes, 0.1 mM ascorbate, 26.5 mM LiBr, 0.7 mM Tris, and 35 μM EDTA, pH 7.0. MalNEt was added to 20 μM, a 7.7 molar excess over enzyme. Initial specific activity of the enzyme–phospholipid complex varied in different experiments from 45 to 89. After reactivation, initial activity of the apoenzyme was 41–80. (B) Stoichiometry of inactivation. The data have been replotted to correlate derivatization by MalNEt with loss of activity. The stoichiometry was unaffected in the range of specific activity 41–89. Lines were drawn for each set of data according to the method of least squares. Apoenzyme (×); enzyme–lipid complex (⊙).

activity when 1 equiv of label is incorporated. The solid line was drawn according to the method of least squares and is not significantly different from a line through 100% and 1.0 mol/mol which would be obtained for a stoichiometry of one.

The active enzyme–phospholipid complex used in most of these experiments was formed by reactivation of the apodehydrogenase with phospholipid vesicles of mitochondrial phospholipid. D-β-Hydroxybutyrate dehydrogenase can also be reactivated, though less efficiently, with aqueous microdispersions of lecithin alone (Nielsen & Fleischer, 1973; Isaacson et al., 1979). Enzyme–phospholipid complexes can also be formed with phospholipid dispersions devoid of lecithin, but such complexes are inactive. Each of the several forms of the enzyme (apoenzyme, enzyme–MPL complex, enzyme–lecithin complex, and enzyme complexed with nonactivating phospholipids) has the same inactivation stoichiometry: the enzyme is inactivated by derivatization with 1 equiv of MalNEt (Table I).

The apodehydrogenase is inactivated by air oxidation, which is the reason for routinely including DTT in the medium for storage. When air is passed over a solution of the apodehydrogenase in the absence of sufficient DTT, enzymic activity is lost. Subsequent reaction with an excess of MalNEt gave reduced labeling (Table I).

Table I: Derivatization of D-β-Hydroxybutyrate Dehydrogenase with *N*-Ethylmaleimide

	mole ratio (mol of MalNEt/mol of enzyme)
Inactivation Stoichiometry[a]	
apoenzyme[b]	1.0
enzyme-MPL complex[b]	1.0
enzyme-lecithin complex[c]	0.9
enzyme-phosphatidylethanolamine–diphosphatidylglycerol complex[d]	0.9
Maximum Derivatization with Excess MalNEt[e]	
apoenzyme[f]	1.7
enzyme-MPL complex[f]	1.9
apoenzyme inactivated by oxygen[g]	0.4
apoenzyme denatured by dodecyl sulfate[h]	5.1

[a] The stoichiometry of inactivation was determined by the graphic method as shown in Figure 1B. [b] Reaction conditions were as described in Figure 1. The results were the same over a range of protein concentrations (90–530 μg/mL) and MalNEt concentrations (7–180 μM). [c] The enzyme-lecithin complex was preformed by incubating for 15 min at room temperature by adding apoenzyme (110 μg/mL final concentration) to mitochondrial lecithin (300 μg of lipid phosphorus/mg of enzyme) in 5 mM Hepes, 0.2 mM ascorbate, 13 mM Tris, 0.67 mM EDTA, and 33 mM LiBr, pH 7.0. Initial specific activity of the enzyme–lecithin complex was 27. MalNEt was added to 8.3 μM (2.4-fold excess). [d] The enzyme-phosphatidylethanolamine-diphosphatidylglycerol complex was preformed as with lecithin except that the lipid (a mixture of 10:1 mitochondrial phosphatidylethanolamine/diphosphatidylglycerol) to protein ratio was 500 μg of P/mg of protein. Initial specific activity of this complex was 9 after reactivation with MPL. [e] The maximal derivatization was determined as the amount of MalNEt bound after prolonged incubation times with a large excess of reagent. [f] Several concentrations of protein (93–590 μg/mL) and MalNEt (120–2600 μM; 40–140-fold excess) were used with similar results. The reaction was allowed to proceed 3 h at 0 °C with other conditions as described for the inactivation stoichiometry (see footnote *c*). After this reaction time, 90–99% of the enzyme was inactivated. [g] Oxygen inactivation was carried out with 100 μg/mL apodehydrogenase which was stirred under oxygen for 5 h at room temperature in the buffer described in Figure 1. The enzyme was 85% inactivated by this treatment. MalNEt was then added to 180 μM (60 mol/mol of protein) and allowed to react for 3 h. [h] Apodehydrogenase (610 μg/mL) was dialyzed under nitrogen against 100 mM sodium phosphate, 50 μM EDTA, and 2% dodecyl sulfate, pH 7.0. MalNEt was added to 1.2 mM (60 mol/mol of protein) and incubated under nitrogen for 12 h at room temperature. Unbound MalNEt was removed by dialysis.

Derivatization of the enzyme in the presence of a large molar excess of MalNEt (140-fold) proceeds to a maximum incorporation of 2 mol of label/mol of enzyme (Table I). The same end point is reached for both apodehydrogenase and the enzyme–phospholipid complex. Denaturation with dodecyl sulfate results in labeling of five cysteine residues, in agreement with amino acid analyses (Bock & Fleischer, 1975; S. C. Brenner, S. Fleischer, and K. Mann, unpublished studies). This suggests that the native enzyme contains no disulfide bridges and that two cysteine residues are "accessible" to derivatization with MalNEt, whereas three are "inaccessible".

Coenzyme Protection against Inactivation. The inactivation stoichiometry of 1 mol of label/mol of enzyme implies derivatization at or near the active center of the enzyme. The labeling experiments were repeated in the presence of known cofactors and inhibitors. Since coenzyme (NAD or NADH) is bound only after the enzyme has been complexed with lipid

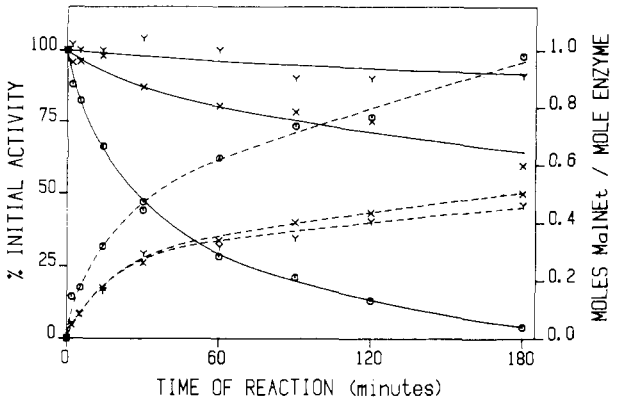

FIGURE 2: Protection of enzyme from inactivation and labeling by coenzyme. [^{14}C]MalNEt (7 μM final concentration) was added to enzyme–MPL complex (100 μg of protein/mL; 3.2 μM) in 5 mM Hepes, 50 mM LiBr, and 0.1 mM EDTA, pH 7.0. Initial specific activity was 90. Unprotected (⊙); plus 2 mM NADH (×); plus 4 mM NAD and 4 mM 2-methylmalonate (Y). Enzymic activity (—); incorporation of label (---).

(Gazzotti et al., 1974), these experiments were carried out only on the active enzyme–phospholipid complex. The enzyme is competitively inhibited with respect to β-hydroxybutyrate by 2-methylmalonate (Tan et al., 1975). NAD and 2-methylmalonate together effectively protect the enzyme–phospholipid complex from inactivation, and NADH alone is slightly less effective (Figure 2). When the enzyme is fully protected against inactivation, it is still possible to incorporate 0.5 mol of label, suggesting that labeling under these conditions occurs at a different site from that involved in inactivation of the enzyme.

Derivatization of the Enzyme Blocks NADH Binding. When the unprotected enzyme is derivatized with MalNEt, it loses the ability to bind coenzyme. Binding of NADH to D-β-hydroxybutyrate dehydrogenase can be measured by energy transfer from the intrinsic fluorescence to bound NADH (Gazzotti et al., 1974). Figure 3A shows the effect of several levels of derivatization on fluorescence binding titrations with NADH. The fluorescence of free NADH is minimized by excitation at its absorbance minimum at 290 nm (Velick, 1958). When the enzyme is derivatized with MalNEt, which blocks the coenzyme binding site, the binding of NADH can be described by

$$[\mathrm{NADH_b}] = \frac{\alpha n P[\mathrm{NADH_f}]}{K_d + [\mathrm{NADH_f}]} \quad (1)$$

where α is the fraction of competent sites, i.e., those which can still bind NADH after partial derivatization, n is the number of binding sites on each protein molecule, P is the total protein concentration, K_d is the NADH dissociation constant and the subscripts b and f refer to bound and free NADH concentrations, respectively.

The data in Figure 3A can be used to determine the NADH dissociation constant and limiting fluorescence when all sites are saturated, by correction for fluorescence of free NADH and approximation of free NADH by the total NADH concentration. The binding equation (1) can be modified and rearranged in double-reciprocal fashion

$$\frac{1}{F_b} \simeq \frac{K_d}{\varphi_b \alpha n P} \frac{1}{[\mathrm{NADH_T}]} + \frac{1}{\varphi_b \alpha n P} \quad (2)$$

where F_b is the fluorescence of bound NADH and φ_b is a factor relating the concentration of bound NADH to its fluorescence. Figure 3B shows the data treated according to eq 2. The intercept of the y axis is the reciprocal of the limiting

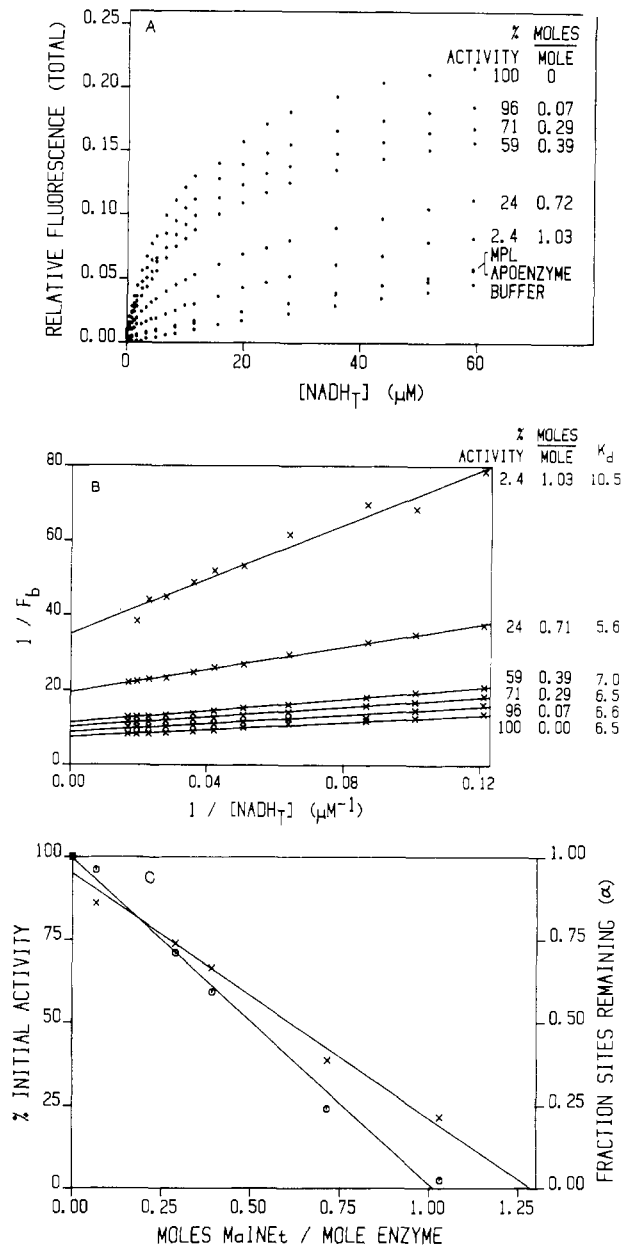

FIGURE 3: Binding of NADH to the derivatized enzyme. (A) Enzyme–phospholipid complex was reacted with MalNEt as described in Figure 1. Aliquots were removed after several time intervals and the unreacted MalNEt was quenched with excess DTT. The samples were then dialzyed overnight against 3 × 100 volumes of 20 mM Mops, 0.1 M NaCl, 1 mM EDTA, and 5 mM DTT, pH 7.2. Fluorescence energy transfer from enzyme to bound NADH (λ_{ex} = 290 nm, λ_{em} = 455 nm) was measured in a Hitachi Perkin-Elmer Model 512 spectrofluorometer. The initial protein concentration was 1.43 μM. Control titrations were carried out for three conditions: (1) buffer; (2) underivatized apodehydrogenase; (3) MPL at the same concentration as in the derivatized samples (3.2 μg of lipid phosphorus/mL). Each titration curve is identified with the activity relative to the unreacted control and by the mole ratio of bound MalNEt to enzyme. (B) The reciprocal of the fluorescence due to bound NADH is plotted vs. the reciprocal of the total NADH concentration according to eq 2. The bound fluorescence was calculated by subtraction of a constant background and of the fluorescence due to free NADH. Each line is identified by the activity and amount of label as in part A, as well as with the K_d determined as described in the text. (C) The limiting fluorescence at saturation was determined from the intercepts (part B) and the fraction of sites available for binding NADH was determined from eq 3. For comparison, the inactivation stoichiometry is also shown. Stoichiometry of binding inhibition (×); inactivation stoichiometry (⊙). The lines in parts B and C were drawn for each set of data according to the method of least squares.

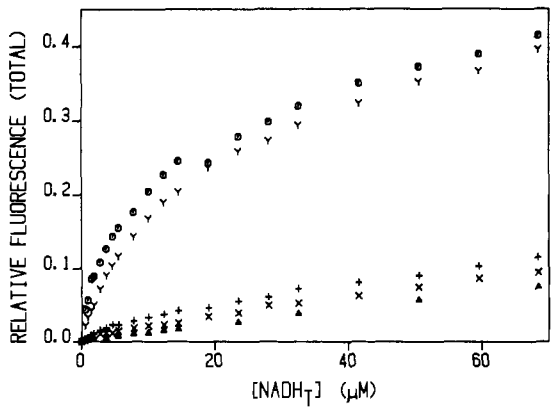

FIGURE 4: Effect of methylmercury and oxidation on coenzyme binding. The enzyme–phospholipid complex was inactivated either by stirring under a stream of oxygen for 26 h or by addition of a twofold molar excess of methylmecury hydroxide. Coenzyme binding was measured by fluorescence titration as described in Figure 3A at a protein concentration of 3.2 μM. NADH was titrated into unmodified enzyme–phospholipid complex (⊙), buffer alone (▲), oxygen inactivated enzyme–phospholipid complex (+), methylmercury-inactivated enzyme–phospholipid complex (×), and methylmercury-treated enzyme–phospholipid complex, reactivated with 1 mM DTT prior to titration (Y).

fluorescence (F_{lim}) at saturation, and the dissociation constant (K_d) was determined as the quotient of slope/intercept. The constant K_d at each level of derivatization indicates that the effect of modification is to change the fraction of available sites rather than to decrease the binding affinity. This allows us to determine the fraction of remaining binding sites at each level of derivatization by comparison of the limiting fluorescence with that of the underivatized sample (for which α = 1) according to eq 3. Figure 3C compares the fraction of

$$\alpha = F_{lim,derivatized}/F_{lim,underivatized} \quad (3)$$

binding sites remaining (α) with the fraction of activity remaining at several levels of derivatization. The inactivation stoichiometry for enzymic activity is 1.0 and for NADH binding is somewhat greater. In view of the complexity of these experiments, the stoichiometries of inactivation and loss of NADH binding capacity are probably not significantly different. It would appear that derivatization of the essential sulfhydryl leads to loss of enzymic function as a consequence of loss of NADH binding.

It may be argued that MalNEt, although not a large reagent, blocks NADH binding by steric hindrance. Methylmercury, an even smaller reagent, reversibly inactivates the enzyme (>95%) when <2 mol are added per mol of enzyme (data not shown). In these experiments, albumin was omitted from the assay mixtures since its presence reverses methylmercury inactivation of the enzyme, undoubtably because it provides sulfhydryls which compete for this reversible inhibitor. Oxygen also inactivates the enzyme, probably by disulfide formation (Table I). In this case, no bulky reagent is added to sterically interfere with coenzyme binding. Both methylmercury and air oxidation also block NADH binding (Figure 4), analogous to derivatization with MalNEt (Figure 3).

Discussion

D-β-Hydroxybutyrate dehydrogenase is a lipid-requiring enzyme with pyridine nucleotide as coenzyme. The lipid-free apodehydrogenase is unable to bind its coenzyme. The ability to bind coenzyme requires formation of the enzyme–phospholipid complex. In the present study, we find that a single sulfhydryl moiety is essential for enzymic function, and, in this regard, D-β-hydroxybutyrate dehydrogenase is similar to some soluble dehydrogenases. However, unique to this enzyme, we find a parallel loss of enzymic function and NADH binding, with complete loss of both capabilities when 1 equiv of sulfhydryl is derivatized. Because derivatization of this group completely prevents coenzyme binding, which is the first step in the ordered sequential reaction (Nielsen et al., 1973), we are unable to determine whether there is also an effect on the catalytic event.

There are five cysteine residues per enzyme polypeptide chain (Table I; Bock & Fleischer, 1975; S. C. Brenner, S. Fleischer, and K. Mann, unpublished studies). Of these, two sulfhydryl groups in the native enzyme are accessible for derivatization with MalNEt. The three remaining sulfhydryls can be derivatized in the presence of dodecyl sulfate, indicating that in the native enzyme they are reduced but inaccessible.

The two sulfhydryl groups which are accessible to derivatization in the native enzyme appear to be nonequivalent in that the essential sulfhydryl can be protected against derivatization in the presence of NADH, or NAD^+ plus methylmalonate, with no loss of enzymic activity. Under these conditions the second accessible sulfhydryl appears to become labeled and is therefore nonessential for catalysis. Thus, it would appear that we have developed methodology to selectively derivatize the only accessible nonessential sulfhydryl in the enzyme with an extrinsic probe with retention of function. This new methodology should be useful for future study of lipid–protein interaction of a functional enzyme using biophysical techniques such as fluorescence and EPR.

The derivatization studies of D-β-hydroxybutyrate dehydrogenase were carried out primarily with *N*-ethylmaleimide. We believe that derivatization is specific for one essential sulfhydryl group which is required for both enzymic activity and NADH binding since (1) derivatization with MalNEt at pH 7 is specific for sulfhydryl groups (Li & Vallee, 1963), (2) the enzyme can be protected from derivatization with MalNEt by pretreating with mersalyl, which is sulfhydryl specific, (3) there is parallel loss of enzymic activity and NADH binding when 1 equiv of sulfhydryl is derivatized, (4) NADH or NAD^+ plus methylmalonate protect against inactivation with MalNEt, and (5) air oxidation or treatment with methylmercury results in loss of coenzyme binding in parallel with inhibition of enzymic activity.

We find that MalNEt blocks enzymic function in both directions (not shown) as would be predicted from the symmetry requirements of an ordered sequential reaction (Nielsen et al., 1973). The effects of MalNEt on coenzyme binding were studied by using NADH rather than NAD because of the tighter binding of reduced coenzyme (Gazzotti et al., 1974). Implicit for an ordered sequential mechanism in which coenzyme must bind before substrate, there can be no enzymic catalysis when coenzyme binding is precluded by derivatization.

The importance of sulfhydryls for D-β-hydroxybutyrate dehydrogenase had been suggested quite early in the study of the enzyme. Wise & Lehninger (1962) recognized that reducing agents were required to maintain activity of the enzyme in rat liver mitochondria. Sekuzu et al. (1963) demonstrated the sensitivity of a detergent-solubilized beef heart enzyme preparation to reagents such as Cd^{2+} and BAL plus arsenite, implying the presence of a functional vicinal dithiol (Fluharty & Sanadi, 1961). Latruffe & Gaudemer (1974) suspected the involvement of an essential sulfhydryl since coenzyme protected against inactivation of the enzyme in rat liver mitochondria by sulfhydryl reagents. We now show, by direct

study on the purified enzyme, the loss of both enzymic activity and coenzyme binding by derivatization with 1 equiv of MalNEt. Although several soluble dehydrogenases have essential sulfhydryl groups, modification of these residues did not prevent coenzyme binding as in the present study [liver alcohol dehydrogenase (Li & Vallee, 1963); yeast alcohol dehydrogenase (Dickinson, 1972); lactate dehydrogenase (Holbrook, 1966)]. Our results suggest that D-β-hydroxybutyrate dehydrogenase is unique, compared to these soluble dehydrogenases, in that both phospholipid and a sulfhydryl moiety on the enzyme are required for coenzyme binding.

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Determination of Polypeptide Amino Acid Sequences from the Carboxyl Terminus Using Angiotensin I Converting Enzyme†

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ABSTRACT: A method for sequence analysis of polypeptides starting at the carboxyl terminus is described that utilizes degradation of the polypeptide into dipeptides with angiotensin I converting enzyme. Dipeptides were identified by gas chromatography–mass spectroscopy. Dipeptide alignment was achieved by replicate digestion of the polypeptide after modification at the carboxyl terminus either by chemical or enzymatic removal of one residue or by addition of a single residue. The addition reaction involved coupling of L-α-aminobutyric acid under conditions described herein which yielded essentially complete conversions. Unlike sequence determination methods that commence from the polypeptide amino terminus, this procedure does not require that a polypeptide have a free amino terminus for successful application. A number of polypeptides with varying chain lengths (up to 49 residues), containing among them most of the common amino acids, have been successfully analyzed in amounts as low as 5 nmol.

Most of the widely applied and well-established methods for the determination of polypeptide primary structure are based on sequential degradations that start from the amino terminus. Although these procedures most frequently utilize variations of the Edman degradation (Edman, 1970), they may also be accomplished by the rapid and sensitive dipeptidyl aminopeptidase (DAP)[1] method (Krutzsch & Pisano, 1977; Seifert & Caprioli, 1978). However, amino-terminal sequence methods fail completely if a polypeptide has a blocked N terminus or give only partial results if the sequence deter-

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[1] Abbreviations used: DAP, dipeptidyl aminopeptidase; C, carboxy; N, amino; DCP, dipeptidyl carboxypeptidase; GC–MS, gas chromatography–mass spectrometry; ACE, angiotensin I converting enzyme; NMM·HOAc, *N*-methylmorpholine acetate; FA-F-G-G, furanacryloyl-L-phenylalanylglycylglycine; Me_3Si, trimethylsilyl; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; DMAC, *N,N*-dimethylacetamide; Abu-O-*t*-Bu, L-α-aminobutyric acid *tert*-butyl ester; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; CPB, carboxypeptidase B; CDI, 1,1′-carbonyldiimidazole; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.