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Inhibition of D(−)-β-Hydroxybutyrate Dehydrogenase by Modifiers of Disulfides, Thiols, and Vicinal Dithiols†

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ABSTRACT: D(−)-β-Hydroxybutyrate dehydrogenase of beef heart mitochondria catalyzes the reversible oxidation of D-(−)-β-hydroxybutyrate to acetoacetate in the presence of NAD. Both the membrane-bound and the soluble forms of the enzyme are inhibited by modifiers of thiols [*N*-ethylmaleimide (NEM) and *p*-(chloromercuri)phenylsulfonate (*p*CMS)], vicinal dithiols [phenylarsine oxide and diazenedicarboxylic acid bis(dimethylamide) (diamide)], and disulfides (sulfite, sulfide, and cyanide). NAD and NADH, but not β-hydroxybutyrate and acetoacetate, protect the enzyme against inhibition by NEM, *p*CMS, phenylarsine oxide, and diamide. As tested with NEM and diamide, the inhibitions caused by mono- and dithiol modifiers were pseudo first order, and the reaction order with respect to the concentration of either inhibitor was unity, thus indicating the modification of a single essential thiol and/or dithiol. Sulfite and sulfide inhibitions appeared to be competitive with respect to β-hydroxybutyrate, with K_i values of 10–15 and about 240 μM, respectively. Sulfite inhibition was uncompetitive with respect to NAD, NADH, and acetoacetate. The above results have suggested the presence in D(−)-β-hydroxybutyrate dehydrogenase of an essential thiol and/or a vicinal dithiol associated with the binding site(s) of NAD and NADH. The inhibition by sulfite, sulfide, and cyanide might be indicative of the presence of an essential disulfide or due to a ternary complex formation involving the enzyme, NAD, and the above nucleophiles.

D(−)-β-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) was first discovered in 1937 by Green et al. (1937) and first isolated in highly purified form by Sekuzu et al. (1963). β-Hydroxybutyrate dehydrogenase is tightly bound to the inner membrane of mitochondria (Grafflin & Green, 1948; Cheldelin & Beinert, 1952; Lehninger & Greville, 1953), with its catalytic site apparently facing the mitochondrial matrix (Gaudemer & Latruffe, 1975). It catalyzes the reversible oxidation of the stereoisomer D(−)-β-hydroxybutyrate to acetoacetate in the presence of NAD. In extrahepatic tissue, but not in liver, acetoacetate is converted to acetoacetyl-CoA by the enzyme succinyl-CoA–acetoacetate thiophorase and is then further converted to acetyl-CoA and completely oxidized through the citric acid cycle. The production of acetoacetyl-CoA is controlled by the mass action effect of the reactants and products of the substrate-level phosphorylation reactions of mitochondria through their modulation of the level of succinyl-CoA (Hatefi & Fakouhi, 1968). The isolated β-hydroxybutyrate dehydrogenase was shown by Sekuzu et al. (1963) and Jurtshuk et al. (1963) to require lecithin for activity and form a complex with the phospholipid and to be activated by thiol compounds and inhibited by Cd^{2+}. There is also additional evidence that both the bovine heart and rat liver enzymes are inhibited by thiol inhibitors (Wise & Lehninger, 1962; Latruffe & Gaudemer, 1975). Nielsen & Fleischer (1973) first isolated the enzyme from bovine heart mitochondria without the use of detergents (Sekuzu et al. used cholate) and showed that it is water soluble, and Bock & Fleischer (1975) subsequently purified the enzyme to homogeneity and demonstrated that it has a subunit molecular weight of 31 500 but can occur in solution in dimeric as well as monomeric form. The interaction of β-hydroxybutyrate dehydrogenase with phospholipids and its insertion into membranes have been studied (Gazotti et al., 1975; McIntyre et al., 1979), and Nielsen et al. (1973) have shown that the kinetics of both the membrane-bound and the soluble enzyme are consistent with an ordered bi-bi mechanism, with NAD being the first substrate to add to the enzyme and NADH the last product to leave. Latruffe & Gaudemer (1974) have arrived at similar conclusions with regard to the rat liver enzyme. Gotterer has partially purified the rat liver dehydrogenase and has studied its lipid requirement (Gotterer, 1967a,b).

The present communication presents data on the inhibitory effects of modifiers of thiols, vicinal dithiols, and disulfides on β-hydroxybutyrate dehydrogenase.

Methods and Materials

Submitochondrial particles were prepared from beef heart according to published procedures (Lee & Ernster, 1968), and apo-β-hydroxybutyrate dehydrogenase was prepared essentially accordingly to Bock & Fleischer (1975). It was activated in the presence of sonicated asolectin plus dithiothreitol as described by these authors, and then excess dithiothreitol was

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removed immediately prior to assay by passing the enzyme preparation through a column of Sephadex G-25. The activity of the enzyme so treated decayed not more than 15% in 2 h.

β-Hydroxybutyrate dehydrogenase activity of SMP[1] was assayed at 37 °C in a solution containing 50 mM Hepes/Tris buffer adjusted to pH 8.1 at room temperature, 5 μM rotenone (in 0.5% final volume of ethanol), and 40–45 μM final concentration of Na^+ ions (adjusted by addition of NaCl). Unless otherwise indicated, the fixed concentrations of NAD and DL-β-hydroxybutyrate were, respectively, 1.0 mM and 10 mM. Acetoacetate reductase activities were measured in the presence of the same concentrations of buffer and rotenone at 37 °C. The fixed concentration of acetoacetate was 2.0 mM and that of NADH 0.2 or 0.3 mM as indicated. In all the assays, enzyme plus variable substrate ± inhibitor (or solvent) were incubated together at 36–37 °C for 2–3 min as indicated in the figure legends, and the reaction was started by addition of the fixed substrate. The "fixed-time preincubation" procedure is described under Results. Sodium sulfite and sodium sulfide solutions were prepared immediately prior to use in water that had been preboiled and gassed with argon. The concentrations of Na_2S solutions were determined with I_2 and back-titration with $Na_2S_2O_3$. Phenylarsine oxide was dissolved in dimethylformamide. The concentration of this solvent in the assay medium was 0.5% (v/v). Diamide and NEM were added as ethanolic solutions at a final ethanol concentration not exceeding 0.5% (v/v). Specific activity is expressed throughout as micromoles of NAD reduced or NADH oxidized per minute per milligram of protein at 36–37 °C. The reduced *minus* oxidized absorbance of NAD at 340 nm used for calculation of activities was 6.22 $mM^{-1} \cdot cm^{-1}$. Protein of the SMP preparations was determined by the biuret method (Gornall et al., 1949) in the presence of 1 mg of deoxycholate/mL.

Sodium DL-β-hydroxybutyrate, lithium acetoacetate, *p*CMS, NEM, and Tris were obtained from Sigma, NAD and NADH from P-L Biochemicals, phenylarsine oxide from Aldrich Chemical Co., asolectin from Associated Concentrates, and diamide and Hepes from Calbiochem. Other chemicals were reagent grade.

Results

Assay conditions, especially the ionic composition and strength of the assay medium, have been shown to affect the kinetics of β-hydroxybutyrate dehydrogenase (Nielsen et al., 1973). Under our assay conditions, which were similar to those of Nielsen et al. (1973), the kinetic constants for β-hydroxybutyrate oxidation (forward) and for acetoacetate reduction (reverse) were derived as follows. Four sets of double-reciprocal (Lineweaver–Burk) plots were obtained each at six variable and six fixed concentrations of the substrates of the forward and reverse reactions. The concentration ranges used were 0.62–20 mM DL-β-hydroxybutyrate, 0.1–2.0 mM NAD, 0.1–2.0 mM acetoacetate, and 0.033–0.2 mM NADH. All the plots gave straight lines, and in each set the lines converged to the left of the ordinate as expected. Replots of slopes and ordinate intercepts of the double-reciprocal plots vs. the reciprocal of the concentrations of the fixed substrate in each case, according to Cleland (1970), gave the following values for the K_m of the substrates: NAD, 0.175 ± 0.009 mM;

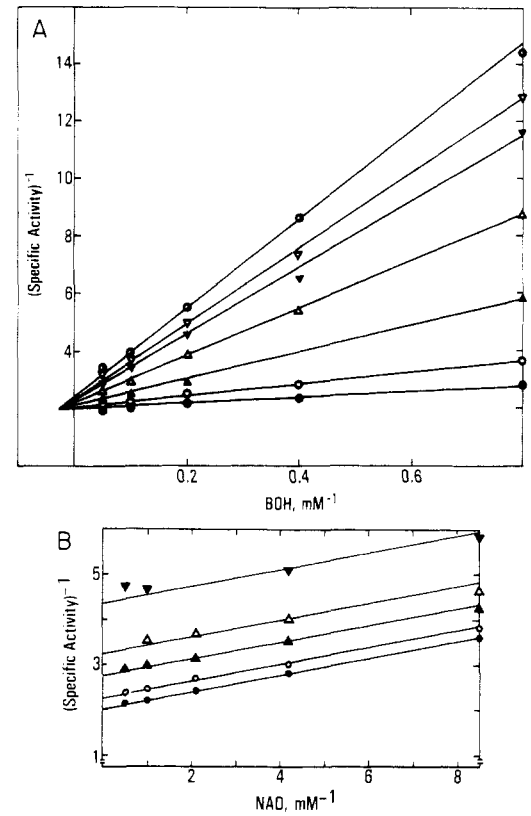

FIGURE 1: Double-reciprocal plots of β-hydroxybutyrate dehydrogenase activity vs. (A) DL-β-hydroxybutyrate concentration at 0 (●), 10 (○), 20 (▲), 60 (△), 80 (▼), 100 (▽), and 120 (◑) μM Na_2SO_3, and (B) NAD concentration at 0 (●), 20 (○), 40 (▲), 80 (△), and 120 (▼) μM Na_2SO_3. SMP (63 μg) was incubated at 37 °C for 1.5 min in the assay mixture containing Na_2SO_3 and the variable substrate, and the reaction was started with 1.0 mM NAD in (A) or 10 mM DL-β-hydroxybutyrate in (B). In (B), lines through data points in the presence of Na_2SO_3 were drawn strictly parallel to the bottom line in the absence of Na_2SO_3 in order to allow better visualization of any deviations of the data points.

β-hydroxybutyrate, 0.8 ± 0.05 mM (i.e., 0.4 ± 0.025 for the active D isomer); NADH, 0.08 ± 0.005 mM; acetoacetate, 0.26 ± 0.023 mM. The V_{max} values for β-hydroxybutyrate oxidation ranged between 0.4 and 0.58 with different preparations of submitochondrial particles, and for acetoacetate reduction between 0.44 and 0.5, both in micromoles per minute per milligram of protein at 36–37 °C. The above values are all very close to those reported by Nielsen et al. (1973) for the beef heart enzyme, with the possible exception of the K_m for acetoacetate for which Nielsen et al. reported a somewhat higher value of about 1 mM. Having thus established that the assay conditions were satisfactory, we proceeded to study the effects of the following compounds on β-hydroxybutyrate oxidation and acetoacetate reduction as catalyzed by both the membrane-bound and the soluble β-hydroxybutyrate dehydrogenase.

Effects of Sulfite and Sulfide. As seen in Figures 1 and 2, sodium sulfite is a potent inhibitor of both β-hydroxybutyrate oxidation and acetoacetate reduction as catalyzed by the membrane-bound enzyme. Similar data were obtained with the enzyme solubilized and partially purified according to Bock & Fleischer (1975). Figures 1B and 2 show that sulfite inhibition was uncompetitive with respect to NAD (or NADH, not shown)[2] and acetoacetate (also as ascertained by Dixon plots of reciprocal specific activity vs. sulfite concentration, plots not shown),[2] but within limits of experimental

[1] Abbreviations used: *p*CMS, *p*-(chloromercuri)phenylsulfonate; NEM, *N*-ethylmaleimide; diamide, diazenedicarboxylic acid bis(dimethylamide); BOH, DL-β-hydroxybutyrate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SMP, submitochondrial particles prepared by sonication of beef heart mitochondria.

[2] Data not shown are available and will be supplied upon request.

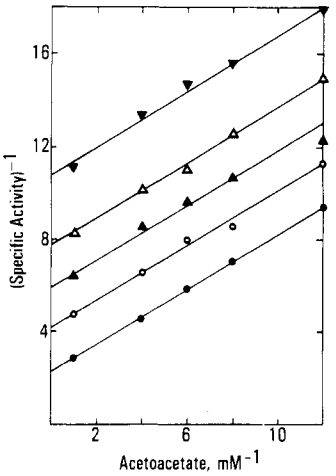

FIGURE 2: Double-reciprocal plots of acetoacetate reductase activity vs. acetoacetate concentration at 0.2 mM NADH plus 0 (●), 20 (○), 40 (▲), 60 (△), and 100 (▼) μM Na_2SO_3. SMP (63 μg) was incubated 3 min at 37 °C in the presence of Na_2SO_3 and variable substrate, and the reaction was started by addition of the fixed substrate. Lines through data points were drawn as in Figure 1B.

accuracy it appeared to be essentially competitive (or perhaps mixed type noncompetitive) with respect to β-hydroxybutyrate (Figure 1A) with a K_i of 10–15 μM as determined from Dixon plots (not shown).[2] While less potent, sodium sulfide also inhibited β-hydroxybutyrate oxidation. Sulfide inhibition also appeared to be competitive with respect to β-hydroxybutyrate concentration, as demonstrated by double-reciprocal plots (data not shown).[2] However, Dixon plots of sulfide inhibition at several fixed concentrations of β-hydroxybutyrate and NAD were biphasic,[2] suggesting more than one site for sulfide interaction. The higher affinity sites indicated K_i values of 240 μM relative to β-hydroxybutyrate and 280 μM relative to NAD. These results suggested that β-hydroxybutyrate interaction with the enzyme is associated with a group, possibly a disulfide (however, see Discussion), which is modified by sulfite or sulfide. Sulfate, thiosulfate, phosphate, arsenate, and arsenite showed little or no effect, while cyanide was inhibitory. Cyanide inhibition with respect to β-hydroxybutyrate was a mixed-type noncompetitive inhibition with a K_i of about 8 mM.

Effects of N-Ethylmaleimide and p-(Chloromercuri)-phenylsulfonate. Figure 3A shows semilogarithmic plots of the inhibition of β-hydroxybutyrate dehydrogenase as a function of incubation time in the presence of increasing concentrations of NEM up to 1.5 μM. Figure 3B shows a similar set of experiments, except that the incubations were performed in the presence of 1.0 mM NAD. It is clear that NEM inhibits β-hydroxybutyrate dehydrogenase with pseudo-first-order kinetics and that NAD offers considerable protection against NEM inhibition. Since this type of activity decay study was difficult to perform and reproduce, especially in the case of other thiol inhibitors we wished to test, we experimented with a different kind of assay in which the possible protective effect of the substrates could be tested. In these experiments, the assay medium was incubated in the presence of one substrate whose concentration was varied, and then enzyme followed immediately by the thiol inhibitor was added to the reaction mixture and allowed to incubate together for a predetermined length of time (e.g., either 2 or 3 min at 37 °C). During this fixed period of time, the enzyme could react with the inhibitor, and the variable substrate present could influence the extent of inhibitor interaction with the enzyme. Then at the end of this fixed period of incubation, the second substrate was added at saturating concentration

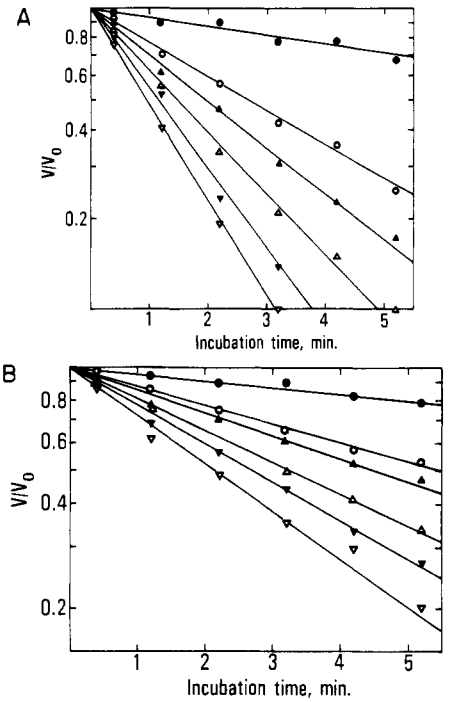

FIGURE 3: Semilogarithmic plots of the inhibition time course of β-hydroxybutyrate oxidation at 0 (●), 0.5 (○), 0.75 (▲), 1.0 (△), 1.25 (▼), and 1.5 (▽) μM NEM in the absence (A) and presence (B) of 1.0 mM NAD. SMP (100 μg) was incubated in buffer plus rotenone for 3 min at 36 °C. At zero time, NEM ± NAD were added together, and incubation continued at 36 °C for the periods of time indicated. Reactions were started by the addition of 10 mM DL-β-hydroxybutyrate + 1.0 mM NAD in (A) or 10 mM DL-β-hydroxybutyrate in (B). V_0, activity at zero time; V, activity at the times indicated.

to start the reaction. When this kind of assay (referred to hereafter as the "fixed-time preincubation" procedure) was used, it was possible to plot the reciprocal of the initial reaction rates vs. the reciprocal of the variable substrate concentration and obtain a family of straight lines for several concentrations of each thiol inhibitor. Figure 4 shows the results of such experiments with NEM and variable concentrations of β-hydroxybutyrate and NAD. It is seen that the straight lines for NAD (or NADH, not shown),[2] but not for β-hydroxybutyrate (or acetoacetate, not shown)[2] converge at the ordinate as though NEM were a competitive inhibitor with respect to NAD. This is, of course, not so, because the inhibition by NEM is irreversible. However, the experimental design is such that *substrate protection* against the inhibitor at several concentrations results in double-reciprocal plots which meet at the ordinate. The fixed-time preincubation procedure was used, therefore, to study the effect of substrates on other thiol inhibitors.

p-(Chloromercuri)phenylsulfonate at high concentrations (~1 μM) reacted too rapidly with the enzyme to allow activity decay studies as a function of time (i.e., similar to those of Figure 3) to be performed conveniently and reproducibly. At lower concentrations (i.e., 0.1–0.5 μM), *p*CMS concentration changed appreciably with time because of its interaction with nonrelevant thiols of the particles. However, when the procedure described above was used, it was found that, similar to the NEM results, NAD and NADH, but not β-hydroxybutyrate and acetoacetate, protected the enzyme against inhibition by *p*CMS.[2]

Effects of Diamide and Phenylarsine Oxide. Diamide and phenylarsine oxide are capable of modifying vicinal dithiols. The former oxidizes dithiols to disulfides, while the latter forms a complex with vicinal dithiols. Figure 5 shows the inhibition of the enzyme in the β-hydroxybutyrate dehydrogenase assay

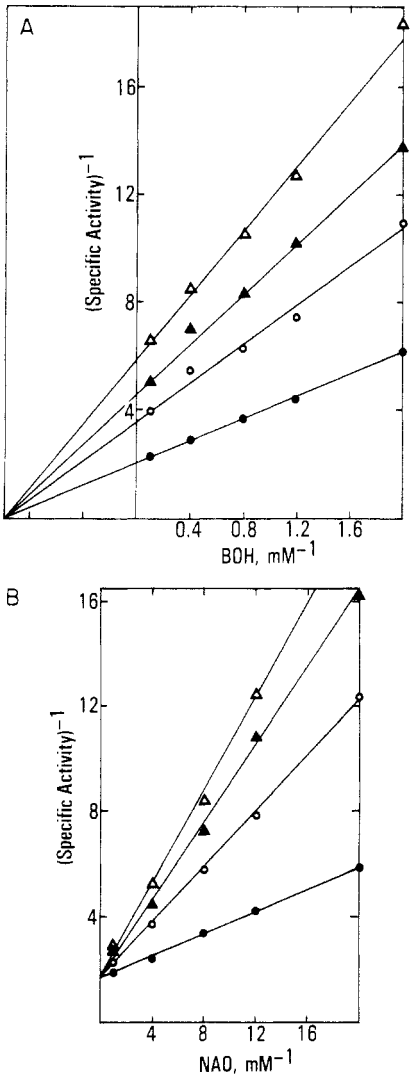

FIGURE 4: Double-reciprocal plots of β-hydroxybutyrate dehydrogenase activity vs. (A) DL-β-hydroxybutyrate concentration at 1.0 mM NAD plus 0 (●), 0.71 (○), 1.0 (▲), and 1.23 (△) μM NEM and (B) NAD concentration at 10 mM DL-β-hydroxybutyrate plus 0 (●), 1.0 (○), 1.41 (▲), and 1.73 (△) μM NEM. Experiments were done by the fixed-time preincubation procedure described in the text. SMP (63 μg) was incubated in the assay mixture precisely 2 min at 37 °C with variable substrate plus NEM, and then the reaction was started by addition of fixed substrate.

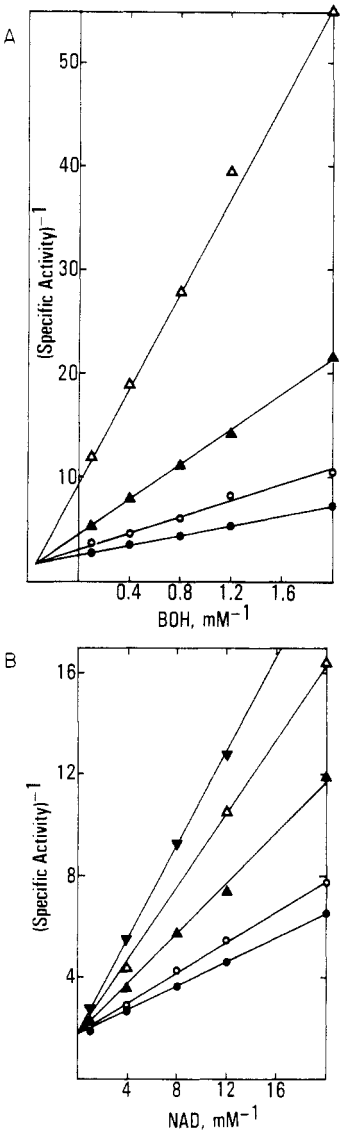

FIGURE 5: Double-reciprocal plots of β-hydroxybutyrate dehydrogenase activity vs. (A) DL-β-hydroxybutyrate concentration at 1.0 mM NAD plus 0 (●), 25 (○), 50 (▲), and 100 (△) μM diamide and (B) NAD concentration at 10 mM DL-β-hydroxybutyrate plus 0 (●), 10 (○), 20 (▲), 50 (△), and 70 (▼) μM diamide. Experiments were done by the fixed-time preincubation (2 min at 37 °C) procedure described in the text and Figure 4 in the presence of 63 μg SMP.

(forward reaction) by diamide and the effect of substrates on this inhibition. The inhibition kinetics of the forward reaction were pseudo first order,[2] and as seen in Figure 5, NAD, but not β-hydroxybutyrate, protected the enzyme against inhibition by diamide. NADH also offered protection, but analysis of the data was complicated because in the presence of diamide NADH appeared to undergo nonenzymatic oxidation at an appreciable rate. However, the data for phenylarsine oxide showed clearly that again both NAD and NADH, but not β-hydroxybutyrate and acetoacetate, protect the enzyme, respectively, in the forward and the reverse reactions against inhibition by this dithiol inhibitor.[2]

Thus, the data of Figures 3–5 indicate (a) that β-hydroxybutyrate dehydrogenase contains a vicinal dithiol, (b) that at least one sulfhydryl group is essential for activity, (c) that the enzyme is protected against inhibition by sulfhydryl modifiers in the presence of NAD or NADH, but not in the presence of β-hydroxybutyrate or acetoacetate, (d) that NAD and NADH also protect against inhibition by modifiers of vicinal dithiols, and (e) that points c and d suggest that either the essential monothiol is part of the vicinal dithiol modified by diamide and phenylarsine oxide or the vicinal dithiol is also essential for coenzyme binding and catalysis. With regard to the latter possibility, the logarithm of the pseudo-first-order rate constants obtained for NEM (Figure 3) and diamide[2] were plotted against the logarithm of the corresponding inhibitor concentrations in order to estimate the reaction orders with respect to inhibitor concentration. It is seen in Figure 6 that in the case of both the monothiol inhibitor, NEM (±NAD), and the dithiol modifier, diamide, the reaction orders were unity, thus indicating that modification of only one thiol (or one vicinal dithiol) is sufficient for enzyme inactivation.

Since the results of Figures 1–5 suggested the presence of a dithiol and possibly a disulfide in β-hydroxybutyrate dehydrogenase, it was of interest to determine whether the dithiol was present in the enzyme before its interaction with substrates, or if the latter interaction resulted in the production of at least one thiolate group from the disulfide. Inhibitory effects observed when the enzyme was treated with diamide in the absence of substrates, and then diluted into the assay medium not containing diamide (Figure 6, right panel), were suggestive that a vicinal dithiol distinct from the disulfide

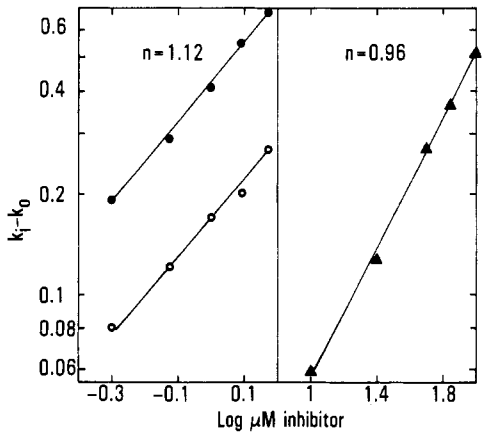

FIGURE 6: Logarithmic plots of the pseudo-first-order rate constants of the plots of Figure 3 and similar plots for diamide (not shown)[2] vs. inhibitor concentrations. (●) NEM (Figure 3A); (○) NEM + NAD (Figure 3B); (▲) diamide. k_0 and k_i are pseudo-first-order rate constants in the absence and presence of inhibitor, respectively, n is the slope of lines indicating reaction order with respect to inhibitor concentration.

might be present in the enzyme. However, a more direct test was also done, as reported in the following paper (Phelps & Hatefi, 1981). Treatment of submitochondrial particles with diamide in the absence of added substrates resulted in inhibition. This inhibition was completely reversed when excess diamide was removed by sedimentation of the particles by centrifugation, and the still-inhibited particles treated with dithiothreitol.

Discussion

It has been shown in the present study that modifiers of monothiols, (NEM, *p*CMS), vicinal dithiols (diamide, phenylarsine oxide), and disulfides (sulfite, sulfide) all inhibit the membrane-bound D(−)-β-hydroxybutyrate dehydrogenase assayed in the direction of β-hydroxybutyrate oxidation (forward) as well as in the direction of acetoacetate reduction (reverse). Although the results have not been shown, the soluble enzyme was also prepared according to the procedure of Bock & Fleischer (1975) and checked in the direction of β-hydroxybutyrate oxidation with regard to the effects of sulfite, NEM, and diamide. The results were essentially the same as those reported above for the membrane-bound enzyme. However, for the purpose at hand, the membrane-bound enzyme was more convenient for us to work with because the soluble preparation was obtained in low yield and its activity was not stable when repeatedly frozen and thawed. Furthermore, because of its requirement for added phospholipids, the use of the soluble enzyme introduced another variable in our assays, which we preferred to avoid now that the inhibition kinetics of a monothiol, a dithiol, and a disulfide modifier were found to be the same for both the membrane-bound and the soluble forms of the enzyme.

Studies with NEM and *p*CMS have shown that β-hydroxybutyrate dehydrogenase contains a thiol group essential for catalysis in both the forward and reverse direction and that NAD or NADH, but not β-hydroxybutyrate or acetoacetate, protect the enzyme against inhibition by the above reagents. The reaction order with respect to NEM concentration in the presence and absence of 1 mM NAD was found to be unity, thus indicating that modification of a single sulfhydryl group was sufficient to render the enzyme inactive. Results with modifiers of vicinal dithiols, namely diamide and phenylarsine oxide, were essentially the same, except for indicating that β-hydroxybutyrate dehydrogenase contains a vicinal dithiol at or near the NAD(H) binding site. Thus, similar to NEM, the inhibition by diamide was shown to be pseudo first order, and the enzyme could be protected against inhibition by the dithiol modifiers by NAD or NADH, but not by β-hydroxybutyrate or acetoacetate. Once again, the reaction order with respect to inhibitor concentration (diamide) was found to be unity, thus indicating that a single dithiol modification was associated with the inhibition kinetics observed. These results also suggested that the essential monothiol modified by NEM or *p*CMS and protected by NAD(H) might be a member of the vicinal dithiol modified by diamide and phenylarsine oxide and similarly protected by NAD(H). Evidence regarding this point is presented in Phelps & Hatefi (1981). It should be added that the fact that β-hydroxybutyrate and acetoacetate did not protect the enzyme against the monothiol and dithiol modifiers is fully consistent with the bi-bi mechanism of the enzyme. However, the observation that NAD or NADH alone (i.e., in the absence of a carboxylic acid) offered considerable protection suggests that the dithiol is at or near the NAD(H) binding site.

The inhibitory effects of sulfite and sulfide suggest that β-hydroxybutyrate dehydrogenase might also contain an essential disulfide, which reacts, for example, with sulfite to yield $^-$S–E–SSO$_3^-$. The inhibitions by sulfite and sulfide appeared to be competitive (or possibly mixed type noncompetitive in the case of sulfite) with respect to β-hydroxybutyrate, while in the case of sulfite the inhibition was uncompetitive with respect to NAD, NADH, and even acetoacetate. Thus, it appeared that the group in the enzyme active site modified by sulfite might be required for binding of β-hydroxybutyrate and/or for transfer of reducing equivalents from β-hydroxybutyrate to NAD. If the group modified by sulfite should prove to be a disulfide, then it is difficult to see how an electroneutral disulfide might be involved in the binding of β-hydroxybutyrate. If breaking of the disulfide by sulfite brings about an inactive conformation of the enzyme, then again it would be difficult to reconcile such a mechanism with the observation that sulfite inhibition is competitive or possibly mixed noncompetitive with respect to β-hydroxybutyrate. It should also be considered that sulfite inhibition is clearly uncompetitive with respect to NAD(H) and especially acetoacetate. Therefore, it seems possible that the target for sulfite on the enzyme might be involved in the oxidation of β-hydroxybutyrate but that in the reverse direction the electronic structure of the active site in the presence of NADH and acetoacetate is such that sulfite can no longer compete with the oxidized form of the substrate. In the experiments with sodium sulfide,[2] inhibition of β-hydroxybutyrate oxidation at several fixed concentrations of β-hydroxybutyrate and NAD resulted in biphasic Dixon plots, thus suggesting more than one site of interaction for sulfide. Since sulfide inhibition appeared in double-reciprocal plots to be competitive with respect to β-hydroxybutyrate, it is possible that one site of sulfide interaction is the same as the site of sulfite interaction. What the second site of sulfide interaction might be is difficult to consider at this time. However, the low slopes of the second phases of the Dixon plots[2] suggest the possibility of interfering contaminants in our preparations of sodium sulfide, even though the crystal surfaces were carefully cleaned before use, and only fresh solutions in water boiled and gassed with argon were used.

In addition to a disulfide, sulfite can react with a sulfhydryl group in the presence of molecular oxygen as shown in eq 1.

$$\text{E–SH} + \text{SO}_3^{2-} + {}^1/_2\text{O}_2 \rightarrow \text{E–SSO}_3^- + \text{OH}^- \quad (1)$$

Furthermore, since the enzyme has been shown to contain a

vicinal dithiol, $E\text{-}SSO_3^-$ could presumably further react with a second sulfhydryl group to produce $E(S)_2 + HSO_3^-$. Consequently, the oxidation of the enzyme dithiol to disulfide, in the same manner as brought about by diamide, would result in inhibition. However, the latter reaction cannot be involved because (a) sulfite inhibition is reversible by dilution of the enzyme–sulfite mixture, and (b) if this mechanism were operative, one would have expected sulfite inhibition to be protectable by NAD(H), as shown for the inhibition of diamide. Nevertheless, we have checked the possibility shown in eq 1 by studying the inhibitory effect of sulfite on β-hydroxybutyrate oxidation in the presence of NAD and rotenone-treated submitochondrial particles under aerobic and anaerobic conditions. The concentration of sulfite was adjusted to result in about 80% inhibition under aerobic conditions. A duplicate experiment run under anaerobic conditions showed exactly the same degree of inhibition. This result is not in agreement with eq 1 because according to this mechanism absence of oxygen should have resulted in a lesser degree of inhibition by sulfite. As shown by Phelps & Hatefi (1981), beef heart β-hydroxybutyrate dehydrogenase also appears to contain an essential histidyl residue, and hydrogen bonding between a sulfhydryl group and an imidazole nitrogen to give R-S-H···N= has been postulated in the case of other enzyme active sites (Whitehead & Rabin, 1962). However, such a structure would not be expected to interact with sulfite. Another important possibility is adduct formation between sulfite (or sulfide) and NAD, a reaction which is known to result in enzyme inhibition by ternary complex formation involving sulfite, NAD, and enzyme (Parker et al., 1978; Hug et al., 1978). This possibility agrees with the observation that inhibition by sulfite of acetoacetate reduction by NADH was uncompetitive with respect to acetoacetate or NADH concentration. However, it does not agree with the finding that in the forward reaction sulfite inhibition was also *uncompetitive* with respect to NAD concentration. Nevertheless, we feel that inhibition by sulfite–NAD adduct is a strong possibility and should be explored with the purified enzyme so that spectral changes and the effects of pH and UV irradiation can be studied with greater accuracy [see Parker et al. (1978)]. Finally, although inhibitions by sulfite, sulfide, and cyanide are often indicative of modification of a disulfide group, it might be fair to add that sulfite at concentrations of about 10^{-4} M is a potent inhibitor of rhodanase (Volini & Wang, 1973), which does not appear to contain a disulfide (Forbo, 1962). However, in this case, sulfite is a product of the interaction of thiosulfate with cyanide, as catalyzed by rhodanase, to form thiocyanate and sulfite. Furthermore, the cationic site of the enzyme has been shown to bind, in addition to the above substrates and products, other anions such as acetate, formate, and sulfate (Wang & Volini, 1973). In the case of β-hydroxybutyrate dehydrogenase, however, sulfate, phosphate, arsenate, arsenite, bicarbonate, and even thiosulfate showed little or no inhibition, while cyanide caused inhibition only at millimolar concentrations.

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