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Inactivation of D-3-Hydroxybutyrate Dehydrogenase by Fumaroyl bis(methyl phosphate)†

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Abstract—Fumaroyl bis(methyl phosphate) reacts with the NADH-dependent enzyme, D-3-hydroxybutyrate dehydrogenase, leading to irreversible inactivation. The bifunctional reagent cross-links the subunits of the enzyme. The inactivation is subject to saturation and protection by substrate, consistent with the reaction occurring at the active site. The stoichiometry of inactivation indicates two active sites undergo reaction with each equivalent of reagent. These results indicate that the dimeric enzyme has contiguous active sites. The reagent is likely to react with an active site lysine, consistent with previous suggestions.

Introduction

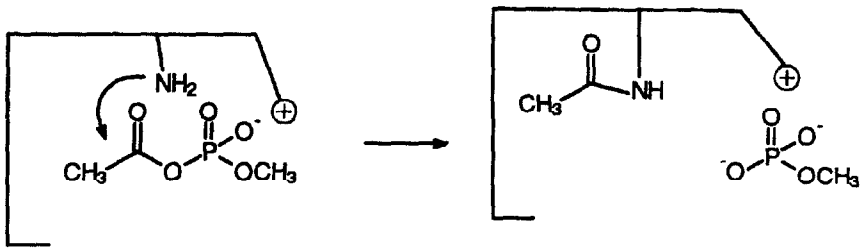
Jones and his co-workers have shown the importance of defining spatial relationships within an enzyme through the systematic evaluation of a diverse group of substrates where the enzyme will tolerate considerable structural variation in this group.[1] For enzymes with a narrow range of substrates, alternative approaches are needed to answer specific structural questions. For example, bifunctional chemicals which permit site-directed protein modification are very useful in defining specific spatial relationships within a protein.[2] In this paper we describe the reactions of a bifunctional anionic acylating agent with an enzyme which has a very narrow range of substrates, D-3-hydroxybutyrate dehydrogenase (HBDH). This enzyme, (EC 1.1.1.30) is a soluble oxidoreductase which catalyzes the stereospecific reduction of β-ketocarboxylic acids. It has been isolated from a variety of sources including animal tissues and bacteria. Although all the enzyme preparations catalyze the same reaction, they have different physical and chemical properties.

Studies of the enzyme from *P. lemoignei* have suggested that the active site contains a cationic moiety which binds the carboxylate group of the substrate.[3,4] It has also been demonstrated that there is a second group in the active site that interacts with the carbonyl group of the ketoacid substrate and with the corresponding hydroxyl group of the hydroxyacid substrate.[3] Based on this model of the active site, Kluger and Tsui[5] developed methyl acetyl phosphate as a substrate analog. This reagent was shown to be a reversible competitive inhibitor and an irreversible inactivator of the enzyme. The behavior was dependent upon the pH and the amount of coenzyme in the incubation mixture. At low pH the inactivation is slow and methyl acetyl phosphate is a competitive inhibitor with respect to acetoacetate (K_i = 10 mM). At higher pH, in the absence of NADH, methyl acetyl phosphate inactivates the enzyme rapidly. The pH dependence for inactivation shows an apparent pK_a = 8.2. Further studies with various specific modifying reagents have demonstrated that the essential group being acylated is an amino function.[7] D-3-Hydroxybutyrate does not protect the enzyme from inactivation and only modest protection is provided by acetoacetate. This observation is consistent with the ordered binding kinetic mechanism reported earlier.[3] NAD, NADH and ADP slowed the rate of inactivation. In the presence of large amounts of NADH the inactivation is biphasic suggesting that two molecules of NADH may bind to two different subunits of the enzyme. In no case was complete protection afforded by a coenzyme or substrate alone.[6]

Methyl acetonylphosphonate (MAcP) is a competitive inhibitor towards acetoacetate.[3] It protects the enzyme from inactivation only in the presence of NADH. The combination of MAcP with ADP did not afford protection from inactivation. If inactivation was due to acylation of sites outside the active site, then protection would not be provided by the competitive inhibitor. Based on these observations, Kluger and Tsui proposed the mechanism shown in Scheme I for the inactivation of the enzyme by methyl acetyl phosphate. These studies of the interaction of methyl acetyl phosphate with HBDH suggested that this reagent may be useful as a general acetylating agent of nucleophilic groups on proteins which are adjacent to cationic sites. Later work by Manning and co-workers showed that methyl acetyl phosphate shows a high degree of predictable selectivity for certain amino groups in human and bovine hemoglobin,[8-10] consistent with the pattern found for D-3-hydroxybutyrate dehydrogenase.

The synthetic method reported for methyl acetyl phosphate could not be extended to give more complex acyl phosphate esters. However, since then, a more general route has been developed and a variety of materials have been prepared. The bifunctional analogs serve as potential site-directed cross-linkers which have many potential applications. In this paper we report a detailed study of the interaction of fumaroyl bis(methyl phosphate) (FBMP) with D-3-hydroxybutyrate dehydrogenase. Since the protein is dimeric, the material appears to be a possible active site-directed cross-linker and inactivator.

†This paper is dedicated to Bryan Jones on his sixtieth birthday.



Scheme I.

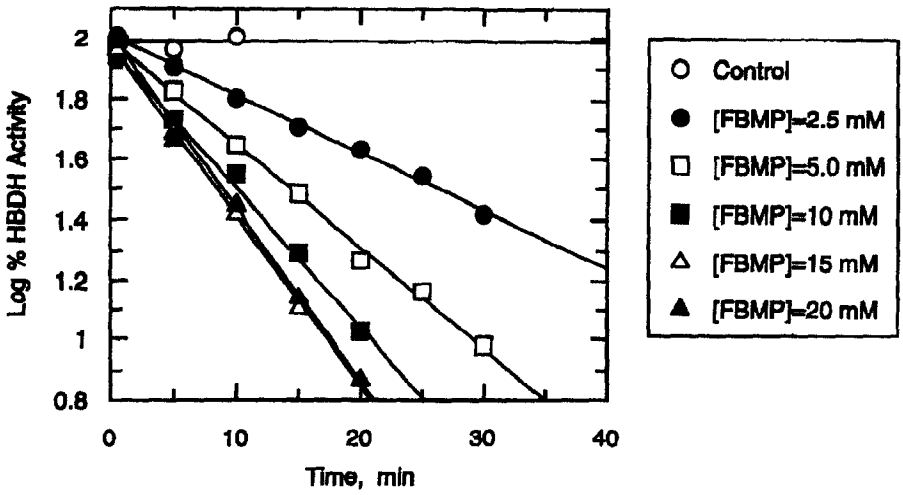
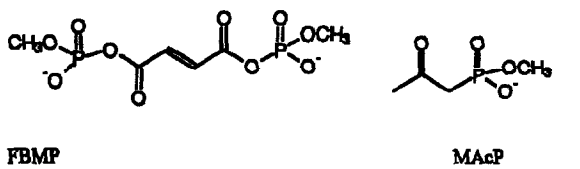


Figure 1. Inactivation of HBDH by FBMP at 25 °C, pH 7.0, 0.1 M KP_i. Enzyme concentration is 35 units/mL. Concentrations of FBMP range from 0 to 20 mM.



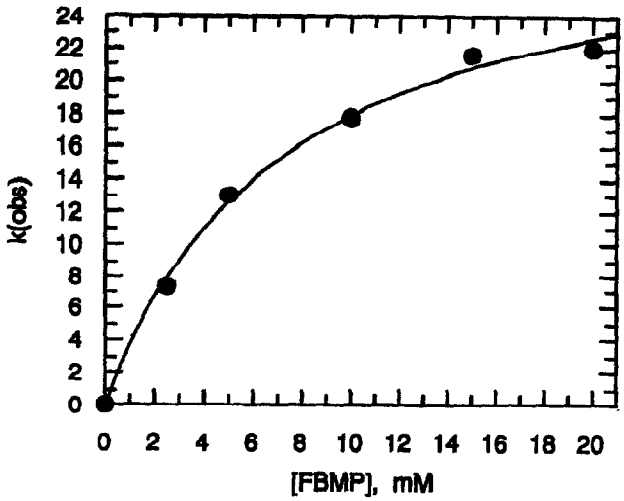


Figure 2. Observed first order rate constant for inactivation of HBDG by FBMP at 25 °C, pH7.0, 0.1 M KP_i.

Results

Chemical modification of HBDH by FBMP

FBMP caused time-dependent loss of HBDH activity. The inactivation followed first order kinetics up to at least 90 % of the reaction. The observed rate constants for inactivation were calculated from the slopes of the semi-logarithmic plot of % activity against time (Figure 1).

Complete loss of enzymatic activity was observed. The inactivation of HBDH by FBMP was irreversible. No activity was recovered when HBDH was treated with FBMP and then dialyzed against 0.1 M potassium phosphate buffer at pH 7.8 for 16 h.

Enzyme–inactivator complex formation

Inactivation of HBDH by FBMP shows saturation kinetics at pH 7.0. The rate of inactivation did not increase linearly with increasing concentration of inactivator; instead it leveled off at high FBMP concentration (Figure 2).

This suggests the initial reversible formation of an enzyme-inactivator complex as shown in Scheme 2.

$$E + FBMP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}FBMP \xrightarrow{k} E\text{-}F$$

$$K_1 = k_2 / k_1$$

$$= [E]\,[FBMP] / [E\text{-}FBMP]$$

Scheme II.

The formation of the reversible complex (E.FBMP), according to the treatment of Kitz and Wilson,[14] is in rapid equilibrium compared to the formation of the irreversibly inactivated enzyme (E–X).

A plot of $1/k_{obs}$ against 1/[FBMP] is linear (Figure 3). At pH 7.0, the intercept on the ordinate is 294 (± 26)s which yields a value of 3.4 (±0.3) x 10^{-3} s^{-1} for the rate constant, k_3. The reciprocal of the intercept on the abscissa gives K_i = 8.9 (±0.1) mM, where K_i is the dissociation constant for the enzyme–inhibitor complex.

Stoichiometry of inactivation of HBDH

The reaction order of the inactivation of HBDH with respect to FBMP can be determined using the treatment of Levy and Ryan.[15] The apparent first order rate constant of inactivation k_{obs} depends on the concentration of FBMP:

$$k_{obs} = k[FBMP]^n,$$

where n is the number giving the order of the reaction with respect to the concentration of FBMP. Taking the logarithm of the equation gives:

$$\log k_{obs} = \log k + n\log\,[FBMP].$$

Thus, a plot of log k_{obs} against log [FBMP] should be linear with a slope of n. At pH 7.0 a straight line with a slope of 0.52(±0.06) was obtained (Figure 4). This demonstrates that 'half of a molecule' of FBMP is involved in the inactivation of the enzyme. Or, in other words, one molecule of FBMP is responsible for the inactivation of two active sites, effectively cross-linking the protein.

Effect of coenzyme on inactivation of HBDH

NADH protects the enzyme from inactivation (Figure 5).

The inactivation follows apparent first order kinetics both in the presence and the absence of the protector. In the presence of a large excess of NADH the amount of protection reaches a limiting value and complete protection is not possible (Figure 6).

Effect of a competitive inhibitor on FBMP inactivation of HBDH

The ability of inhibitors, which are competitive with respect to the substrate, to protect the enzyme from chemical modification usually indicates that the inactivator

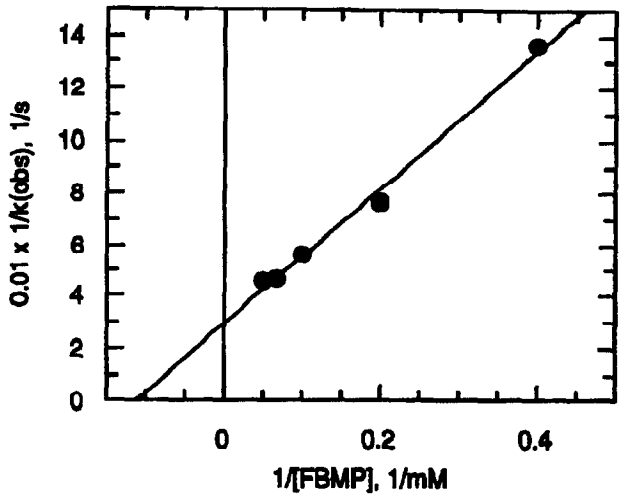


Figure 3. Reciprocal plot of data in Figure 2.

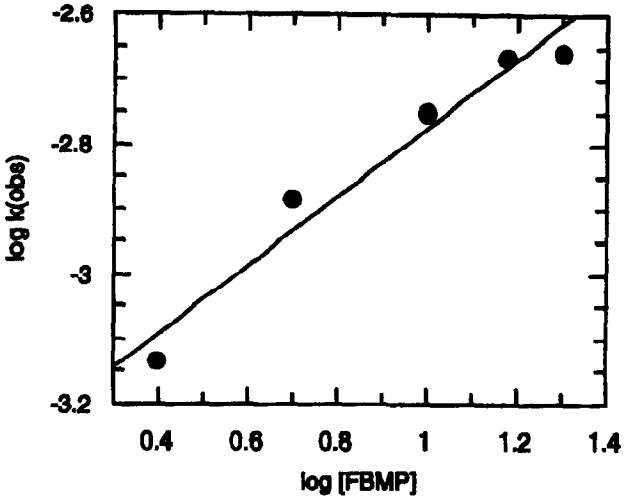


Figure 4. Stoichiometry of inactivation of HBDH by FBMP. Data are from Figure 2. Slope is 0.53 ± 0.06.

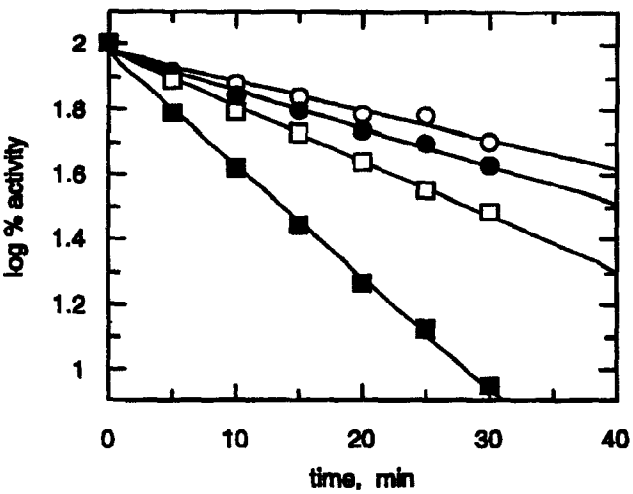


Figure 5. Rate of inactivation of HBDH by 10 mM FBMP as a function of NADH concentration, pH 7.0, 25 °C, 0.1 M KP_i. NADH: 0, 0.1, 0.25, 0.50 mM.

is active site-specific. (It is possible, however, that the binding of the inhibitor to another site brings about a conformational change which leads to the inactivator not being able to bind.)

Methyl acetonylphosphonate (MAcP), is a competitive inhibitor with respect to acetoacetate.[3] It gives only weak protection against inactivation by FBMP in the absence of NADH. However, in the presence of NADH it offers significant protection, presumably due to the formation of the ternary complex:

$$E.NADH + MAcP \rightleftharpoons E.NADH.MAcP.$$

This is in accord with the ordered binding kinetic mechanism of this enzyme.[3] Again, complete protection was not observed (Figure 7).

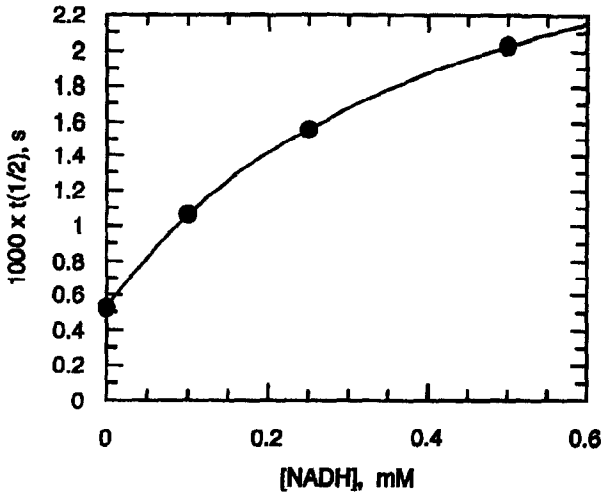


Figure 6. Half-life of inactivation of HBDH by FBMP as a function of NADH concentration, conditions as in Figure 5.

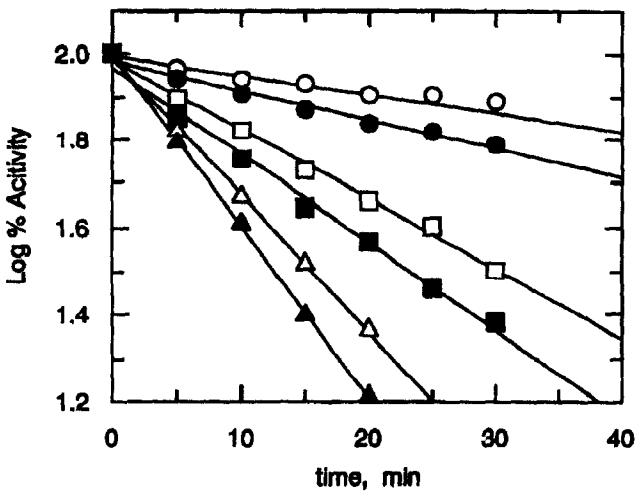


Figure 7. Effect of MAcP on rate of inactivation of HBDH by FBMP, pH 7.0, 25 °C, 0.1 M KP_i, 10 mM FBMP. Additions and plotted points: ○ = 15.0 mM MAcP, 0.1 mM NADH; ● = 5.0 mM MAcP, 0.1 mM NADH; □ = 0.5 mM MAcP, 0.1 mM NADH; ■ = 0.1 mM NADH; Δ = 15.0 mM MAcP; ▲ = no additions.

The ability of NADH and MAcP (in the presence of NADH) to protect HBDH from inactivation indicates that FBMP is an active site-directed reagent for this enzyme.

Electrophoresis

Analysis of HBDH after reaction with FBMP by SDS–PAGE revealed that the subunits of the enzyme had reacted. Lanes containing HBDH reacted with FBMP showed no bands except at the origin even when gels which were 5 % in acrylamide monomer were used or when greater concentrations of urea, SDS or 3-mercaptoethanol were used. This indicates that high molecular weight species (>200,000 Da) are being produced.

Denaturing high performance gel permeation chromatography

When unreacted HBDH was analyzed by HPGPC, a single peak was observed which corresponded to a molecular weight of approximately 50,000 Da. After reaction with FBMP for 10 h and analysis by HPGPC a single peak was observed which corresponded to a molecular weight of 100,000 Da. Analysis of the reaction mixture at 5 min and 74 min after initiation of the reaction between FBMP and HBDH showed peaks corresponding to both 50,000 Da and 100,000 Da.

Discussion

Chemical modification of HBDH by FBMP

FBMP is a bifunctional analogue of methyl acetyl phosphate and has structural homology with acetoacetate. It is likely that because of these features, FBMP has an affinity for the active site of HBDH and forms an E.FBMP complex as evidenced by the rate of inactivation reaching a saturating value at high concentrations of FBMP. The high degree of specificity of this enzyme also implicates active site interactions

Because FBMP is susceptible to nucleophilic attack, suitably placed amino acid residues with nucleophilic side chains will react with it, causing inactivation of the enzyme in a manner similar to that caused by methyl acetyl phosphate.[6] Evidence that FBMP is reacting at the active site is provided by the observation that the coenzyme NADH and the competitive inhibitor MAcP in the presence of NADH afford protection against inactivation.

We can compare the reactivity of FBMP as an acylating agent with other methyl acyl phosphates for which reactions with amine nucleophiles have been studied. Methyl acetyl phosphate has been shown to acetylate an essential lysine residue of pK_a 8.15 in the active site.[6] This pK_a is similar to the pK_a of glycyl-glycine which is 8.17.[16,17] The second-order rate constant for the reaction of glycyl-glycine and phenyl acetyl phosphate (k_2) is 7.6 M^{-1} s^{-1} at 39 °C.[18,19] This yields a value for k_2 of 2.5 M^{-1} s^{-1}

at 25 °C (assuming the same relative change in going from 39 to 25 °C as in the hydrolysis reaction). At pH 7.0 the observed second-order rate constant (k_{2obs}) is given by:

$$k_{2obs} = k_2/(1+[H+]/K_a).$$

A value of k_{2obs} = 0.16 M^{-1} s^{-1} is obtained. For the reaction of FBMP with HBDH, the pseudobimolecular rate constant (k_3/K_i) can be obtained from the reciprocal plot in Figure 3. This yields a value of 0.38(± 0.04) M^{-1} s^{-1} which is twice that estimated for the reaction of phenyl acetyl phosphate with *N*-glycylglycine.[18,19] This value is also approximately twice that found for the reaction of methyl acetyl phosphate with HBDH (k_{2obs} = 0.21 M^{-1} s^{-1}).[6] The slightly increased rate may be due to formation of the E.FBMP complex in which the negative charge of the phosphate group is neutralized to a slightly greater degree than the phosphate group of methyl acetyl phosphate. As a result, the charge density of the phosphate anion is reduced and the attack by the nucleophile is facilitated due to the lowering of the barrier of electrostatic repulsion. The upper limit of this acceleration by charge neutralization is about 2.7 x 10^4 going from methyl acetyl phosphate to dimethyl acetyl phosphate.[20] Thus, the observed rate acceleration does not represent a significant increase in charge neutralization. This supports the proposition that FBMP is modifying an essential lysine residue in a manner analogous to the reaction of methyl acetyl phosphate.

It is interesting to compare the binding constants (K_i) of FBMP and methyl acetyl phosphate to HBDH. FBMP has a higher affinity for the active site of HBDH (K_i = 8.9 mM) than does methyl acetyl phosphate (K_i = 52 mM).[6] It is possible that the additional anionic methyl phosphate group is also binding to a cationic region in the protein such as those sites which are normally involved in the binding of the phosphate groups of NADH or the positively charged nitrogen in NAD. It is also possible that the additional methyl phosphate group is being recognized by the carboxylate binding domain of an adjacent active site. Finally, there may be a hydrophobic interaction between amino acid residues whose normal function is the binding of the nicotinamide ring, and the olefinic function of FBMP. These possibilities seem reasonable considering the effective protection against inactivation provided by the coenzyme NADH.

Cross-linking of HBDH by FBMP

The stoichiometry of inactivation of HBDH by FBMP indicates that one molecule of FBMP is responsible for the inactivation of two active sites. This means that FBMP is capable of cross-linking the active sites of different subunits. Attempts to demonstrate cross-linking by SDS–PAGE were unsuccessful because the protein failed to enter the gel in concentrations that could be visualized by Coomassie Brilliant Blue staining. Generally, most globular proteins are 'well-behaved' on polyacrylamide gels. However, with increasing spherical size or linear extension, mobility is greatly reduced.[21] It is likely that the reaction of FBMP with HBDH produces a form of the protein which is not well-behaved on these gels.

In order to overcome this problem, denaturing high performance gel permeation chromatography (HPGPC) was used. This technique is more sensitive and allows for repeated analysis of the reaction mixture. HBDH from *P. lemoignei* has been characterized by gel permeation chromatography on Sephadex G-150 and found to have a molecular weight of approximately 100,000 Da.[3] SDS gel electrophoresis has been reported to yield bands corresponding to a dimer of two subunits of approximately 50,000 Da each.[3]

HPGPC of native HBDH yielded a molecular weight of approximately 50,000 Da in agreement with the literature. However, after HBDH was incubated for 10 h with FBMP a new single peak appeared in the chromatogram which corresponded to a molecular weight of approximately 100,000 Da. No peaks corresponding to higher molecular weights were observed. This indicates that FBMP is cross-linking the subunits of HBDH.

Significance of cross-linking

The ability of FBMP to cross-link the active sites of HBDH indicates that the active sites must be located in close proximity to each other. Molecular models indicate that the distance between amino groups that can be cross-linked by fumaroyl bis(methyl phosphate) is only about 6.1 Å. This close proximity of active sites may arise if each subunit has an active site and each site is close to the junction of the two subunits or if each subunit contains an active site but the two subunits cross-linked are from different dimers. (The possibility of one of the subunits not containing an active site may be ruled out since no peak corresponding to a molecular weight of 50,000 Da was observed by HPGPC after 10 h of reacting the HBDH with excess FBMP.) If FBMP is cross-linking subunits from different dimers, the active sites must lie close to the surface of the protein so that enough of the unreacted end of the FBMP is available to bind and react at the active site of another subunit. The cross-linking of HBDH by FBMP indicates that the active sites can come into close proximity and are probably located on the outer surface of the protein.

Comparison with the subunit structures of other dehydrogenases

Some dehydrogenases consist of a single polypeptide chain. However, most of the well-known dehydrogenases are oligomeric with most soluble dehydrogenases existing as dimers or tetramers. Trimers are unusual. For example, soluble malate dehydrogenases from pig and cow heart are dimers while yeast alcohol dehydrogenase and pig and dogfish muscle lactate dehydrogenases are tetramers.[22] In most cases, the subunits of a dehydrogenase are identical. In the cases where they are not, as with various isozymes, they are usually of similar size.[22] HBDH is similar in its quaternary structure to other dehydrogenases in that it exists as a dimer and is composed of two subunits of equivalent molecular weight. The enzyme from *P. lemoignei* is not as well characterized as the enzyme isolated from bovine heart mitochondria. The

mitochondrial HBDH, unlike the bacterial preparation, is activated by lecithin.[23] It does, however, in its soluble form, exist as a dimer.[24]

The active site of the mitochondrial HBDH has been characterized by studies with site-specific labeling reagents.[25] Three essential residues have been found: (a) a dithiol at or near the NAD(H) binding site, (b) one or more arginyl residues involved in binding the negatively charged carboxyl group of the substrate, and (c) a histidyl residue which may be involved in hydrogen-bonding with the hydroxyl group of D-3-hydroxybutyrate and the carbonyl group of acetoacetate.[25] The roles of the dithiol and the phospholipid are not well understood but they appear to be involved with nucleotide binding.

This picture of the active site is very similar to that described by Kluger and Tsui[6] for the *P. lemoignei* enzyme except that a histidyl residue is hydrogen-bonding with the substrate rather than a lysine residue. Therefore, despite the mitochondrial HBDH being activated by lecithin while the *P. lemoignei* enzyme is not, these two enzymes do have several features in common. They both exist as dimers with subunits of identical molecular weight, have the same pH-rate profiles, and have very similar active sites.

The reactive sulfhydryl in mitochondrial HBDH is located in the vicinity of the reactive center of the enzyme.[26–28] Spin-label spin-probe measurements.[29] have indicated that the distance of the reactive sulfhydryl in the enzyme is near the nucleotide binding site and is immersed about 9 Å below the membrane surface. This means that a crevice at the surface of the enzyme (protein–water interface) would provide facile access for NAD(H) to its binding site as has been observed for other dehydrogenases.[30]

Thus, if the crevice for reaction is located near the surface, it is reasonable, considering the similarities between dehydrogenases and between the mitochondrial and bacterial preparations of HBDH, to expect the active site of HBDH from *P. lemoignei* to also be near the protein surface. This is in accord with the conclusions of the cross-linking studies of HBDH with FBMP.

Experimental

Materials

D-3-Hydroxybutyrate dehydrogenase from *P. lemoignei* was purchased as a lyophilized powder from Sigma Chemical Co. All other reagents with the exception of NAD were obtained from Sigma. NAD was from both Sigma and Kyowa Hakko Kogyo Co. Ltd. Sodium methyl acetonylphosphonate was prepared according to published procedures.[11] Spectral characteristics were identical to those of the known compound. Fumaroyl bis(methyl phosphate) (FBMP) was synthesized by the method of Kluger *et al.*[12] Data were analyzed using GraFit from Erithacus Software Limited on a MS-DOS computer in Microsoft Windows.

Enzyme assays

D-3-Hydroxybutyrate dehydrogenase. The following solutions were prepared for the assay of D-3-hydroxybutyrate dehydrogenase activity.

A. 0.10 M Tris–HCl buffer, pH 7.8 at 37 °C. 12.11 g of Tris dissolved in 900 mL of water. Equilibrated to 37 °C and pH adjusted to 7.8 with HCl. The solution was then diluted to a final volume of 1 L.

B. 0.05 M NAD solution. 0.1660 g NAD was dissolved in 5 mL water. The solution was neutralized by the addition of solid sodium bicarbonate until effervescence ceased.

C. 0.10 M (D,L)-3-Hydroxybutyrate substrate solution. 0.0630 g (D,L)-3-hydroxybutyric acid, sodium salt, in 5 mL of water.

D. D-3-Hydroxybutyrate dehydrogenase enzyme solution. The lyophilized powder was reconstituted in 0.1 M Tris–HCl buffer, reagent A. The solution prepared typically contained between 0.3 and 0.6 units/mL. The enzyme solution was stored frozen between uses.

Enzyme assays were performed by monitoring the conversion of NAD to NADH at 340 nm at 37 °C. Initial velocity was used to measure the catalytic rates under steady-state conditions. Reagent A (2.60 mL), reagent B (0.10 mL), and reagent C (0.10 mL) were mixed in a 3.0 mL quartz cuvette and equilibrated at 37 °C. Enzyme solution (0.10 mL) was then added to initiate the reaction.

Inactivation studies. Reaction was initiated by addition of enzyme to 1–2 mL of a solution of fumaroyl bis(methyl phosphate) (concentrations ranging from 2.5 to 20.0 mM). The reaction mixture was incubated at 25 °C at pH 7.0 and aliquots were withdrawn for assay of activity. The rate of inactivation was plotted according to observed first-order kinetics with log (% activity) against time. For protection studies either MAcP (0–15 mM) or NADH (0–0.5 mM) was incubated with the FBMP followed by addition of the enzyme.

Electrophoresis. SDS–polyacrylamide gel electrophoresis (PAGE) of D-3-hydroxybutyrate dehydrogenase treated with FBMP was performed to determine if the bifunctional reagent was cross-linking catalytic subunits. Gels were 5 and 7.5 % in acrylamide monomer and contained 0.3 % methylene bis-acrylamide.

Denaturing high performance gel permeation chromatography. The cross-linking of HBDH by FBMP was also investigated by denaturing HPGPC using methodology similar to that of Montelaro *et al.*[13] except 7 M urea was used as the denaturant instead of guanidine hydrochloride or sodium dodecyl sulfate.

The following protein standards (molecular weights indicated) were obtained from Sigma Chemical Co.: myosin from rabbit muscle (205,000), D-3-galactosidase

from *E. coli* (subunit: 116,000), ovalbumin (45,000), and carbonic anhydrase from bovine erythrocytes (29,000).

The HPGPC system consisted of a high performance liquid chromatograph (Waters Associates, Inc. 501 pump) equipped with a U6K injector and a Waters 490E programmable Multiwavelength detector set for 280 nm (0.1 AUFS). The column was a Waters Protein-Pak 300 SW. The eluant was 20 mM sodium phosphate buffer, pH 6.5 containing 7 M urea and was filtered and degassed prior to use. The flow rate was 0.2 mL/min. All procedures were carried out at 25 °C.

Samples were prepared by dissolving the individual protein standards in 20 mM sodium phosphate buffer, pH 6.5 containing 7 M urea and 1 % D-3-mercaptoethanol (to yield a final concentration of 1 mg/mL) and heating at 100 °C for 5 min. Injections varied from 10 to 25 μL.

Unreacted HBDH was dissolved directly in the buffer as described for the protein standards. HBDH was reacted with FBMP; the concentration of HBDH was approximately 2 mg/mL and the concentration of FBMP was 115 mM. The reaction mixture was incubated at 25 °C and aliquots were withdrawn at various time intervals and mixed with an equal volume of 20 mM sodium phosphate buffer containing 7 M urea and 1 % D-3-mercaptoethanol. These were then heated for 5 min, cooled and analyzed by HPGPC.

Acknowledgements

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