Application to Hydrolysis of Acetyl Phosphate Derivatives. The rate of C–O (acyl) cleavage of acetyl phosphate mono- and diesters (acetyl phenyl phosphate,[17] acetyl dimethyl phosphate[20]) is sensitive to the pK_a of the leaving group; the monoanion is cleaved much more slowly than is the neutral compound. Metal ions promote the hydrolysis of the monoanion[21,22] presumably by causing it to resemble the neutral compound. These effects could be due to the fact that a phosphate diester is a much weaker base than a phosphate monoester, and thus is a better leaving group.[22] Our results indicate that the rate-determining step in this reaction is probably addition of water to the carbonyl group to form the hydrated derivative, with expulsion of the leaving group being rapid. Thus, the rate differences are due to the differences in inductive (or resonance) effects of the substituents in the hydration step, and catalysis by metal ions is likely to be the result of promotion of hydration. If the hydrate is only a transition state, then, in effect, expulsion may also be assisted. These matters are under investigation.

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Substrate Analogue Studies of the Specificity and Catalytic Mechanism of D-3-Hydroxybutyrate Dehydrogenase[1]

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Abstract: D-3-Hydroxybutyrate dehydrogenase from *Pseudomonas lemoignei* was analyzed by steady-state kinetics which indicate that an ordered binding mechanism (NADH and then acetoacetate on, hydroxybutyrate and then NAD off) applies. The specificity for binding and the specificity for catalysis of the enzyme to which NADH has bound were determined through the use of carboxylates, phosphonates, and a sulfonate that have structural features in common with acetoacetate. It was found that 3-keto carboxylates, with or without alkyl substituents at the 2 and 4 positions, and acetonylsulfonate are reduced enzymatically. The phosphonate monoanionic analogue of acetoacetate, methyl acetonylphosphonate, is unique in being a competitive inhibitor at pH 7 of reduction of acetoacetate (K_i = 1.65 mM). A variety of anions function as noncompetitive inhibitors. It was shown that V_{max} is independent of the substrate; therefore, it is unlikely that the catalytic step is rate determining. It is proposed that the active site's recognition of the substrate involves a cationic center to which the carboxyl group binds and a hydrogen-bond donor with which the carbonyl group of the substrate or competitive inhibitor can associate. The lack of rotational symmetry in the phosphonate monoester functional group leads to its being bound in a distorted manner compared to molecules that have this symmetry and are substrates. Reduction of the carbonyl group occurs only when a more critically limited spatial arrangement is attained, distinguishing an inhibitor from a substrate. The catalytic process in this system is suggested to involve a transition state for reduction which closely resembles the alcohol and NAD products.

Phosphonates and sulfonates which have structural and electronic features in common with carboxylates can be used to probe the specificity and catalytic mechanism of enzymes that normally catalyze reactions of carboxylates.[2,3] Differences in the inherent reactivity patterns of the various molecular types lead to diverse responses to the catalytic apparatus of an enzyme. In this paper, we report the results of a substrate analogue study of the highly specific bacterial enzyme, nicotinamide adenine dinucleotide-dependent D-3-hydroxybutyrate dehydrogenase (*Pseudomonas lemoignei*)[4] (eq 1). The enzyme is abbreviated HBDH; the cofactors are abbreviated NADH and NAD.

A major advantage of the structural analogue approach to the study of enzymic specificity and catalysis is that definitive information about the accommodations of an enzymic binding site can be obtained for an enzyme for which very little other information is yet available. Thus, once the basic, kinetic-reaction patterns of the enzyme are known, the effects of substrate analogues can be immediately evaluated. This approach is complementary to methods which analyze the protein

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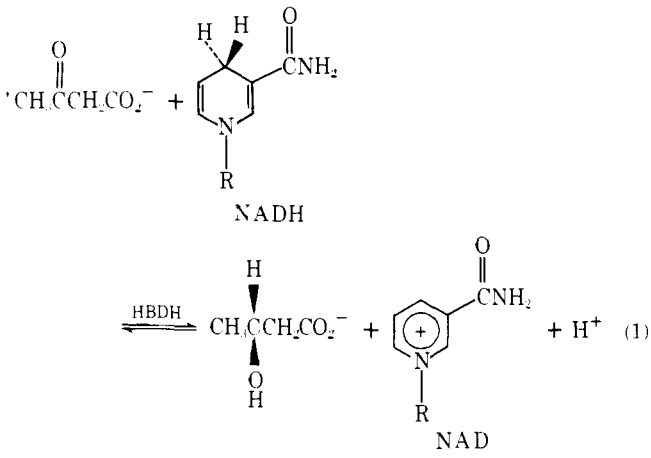

itself. In the case of HBDH very little is known about the protein. The fact that the enzyme was known to be highly specific[4] suggested that the use of substrate analogues could precisely describe the specificity in terms of substrate structure. Furthermore, as a result of such studies we find that information about the catalytic mechanism becomes available as well.

Experimental Section

Materials. Ethyl acetoacetate, ethyl 2-methylacetoacetate, dimethyl methylphosphonate, and trimethyl phosphate were obtained from the Aldrich Chemical Co. and were distilled before use. NADH, NAD, and sodium DL-3-hydroxybutyrate were purchased from the Sigma Chemical Co. and were used without further purification. Tris(hydroxymethyl)aminomethane (Tris) and sodium iodide were purchased from the Fisher Scientific Co., Ltd. All inorganic compounds were reagent grade or the highest commercially available grade. Water was redistilled in a glass apparatus and this was used for preparing all reaction solutions.

The following were prepared according to published procedures: dimethyl acetonylphosphonate,[5] sodium methyl acetonylphosphonate,[2] sodium acetonylphosphonate,[6] sodium acetonylsulfonate,[7] sodium methyl acetylphosphonate,[3] sodium methyl methylphosphonate,[8] methanephosphonic acid,[8] sodium dimethyl phosphate,[8] and lithium acetoacetate.[9] Lithium 2-methylacetoacetate was prepared from its ethyl ester by the same procedure by which lithium acetoacetate is prepared from ethyl acetoacetate.[9]

HBDH from *P. lemoignei* was purchased as a lyophilized powder from Sigma. Purification of the enzyme on Pharmacia Sephadex G-150 did not lead to a change in the enzyme's kinetic parameters or specificity. The species thus characterized has a M_r of ~100 000 daltons.[10] SDS gel electrophoresis[11] gives bands corresponding to a dimer of two ~50 000 M_r subunits.

Methods. All kinetic measurements were carried out by monitoring the conversion of NADH to NAD, or the reverse, by spectrophotometric recording of absorbance at 340 nm due to NADH. A Hitachi Perkin-Elmer Coleman 124 spectrophotometer equipped with a thermostated cell changer, maintained at 37.0 ± 0.10 with a Heto constant temperature circulator, and a Heath recorder was used for all spectroscopic determinations. Initial velocity was used to measure catalytic rates in all cases.

Solutions of enzyme were prepared by dissolving the lyophilized powder in 0.1 M, pH 7.8, Tris hydrochloride buffer. The solution was stored frozen between uses. Enzymic activity of a particular sample of solution was determined using standard concentrations of 2.7×10^{-4} M acetoacetate and 8.5×10^{-5} M NADH, following the rate of decrease of absorbance at 340 nm due to the conversion of NADH to NAD. One unit of enzyme is defined by activity such that 0.375 μmol of NADH is oxidized per milliliter per minute at 37 °C.[4] Each batch of purchased enzyme was determined to be free of contamination by malate dehydrogenase and lactate dehydrogenase by assaying with their respective substrates.

Each substrate analogue or material being tested for inhibitory properties was dissolved in 0.1 M potassium phosphate buffer of the required pH, followed by appropriate dilution with buffer, and the initial rate was measured as described. All lines used in enzyme kinetic analyses were based on a good fit of all data. At least four points were used to determine each line. The range of concentrations used is listed with each result.

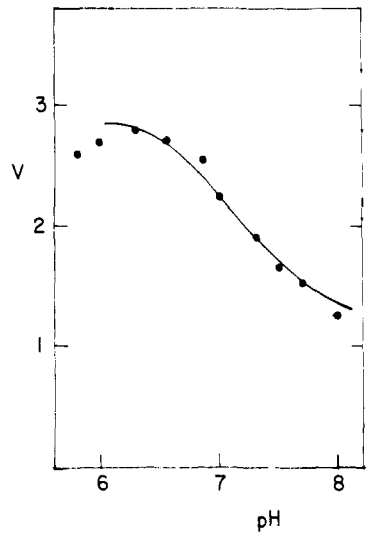

Figure 1. Initial rate of reduction of acetoacetate at 37 °C, 0.1 M potassium phosphate. Concentrations: HBDH, 3.5×10^{-3} unit/mL; acetoacetate, 0.93 mM; NADH, 8.4×10^{-5} M.

Table I. Kinetic Parameters for the HBDH-Catalyzed Reduction of Acetoacetate at 37 °C in 0.1 M Potassium Phosphate Buffer

substrate A	substrate B	pH	$10^5 K_a$,[a] M	$10^4 K_{ia}$, M	$10^3 K_b$, M	$10^7 V$, ms^{-1}
NADH	aceto-acetate	7	1.56	0.71	0.48	1.27
NADH	aceto-acetate	8	14.50	1.48	1.35	4.42

[a] K_a, K_{ia}, K_b, and V according to the definition of Cleland (ref 21).

Results

pH–Velocity Profile. The activity of HBDH in the direction of reduction of acetoacetate as a function of solution acidity is given in Figure 1. The concentration of substrate used is below saturation. The curve in Figure 1 has been drawn according to the equation for titration of an acid of $pK_a = 7.2$. These data, of course, do not imply that an ionization of a single species is occurring.[12]

Kinetic Analysis. Inverse data plots according to the Lineweaver–Burk formulation[13] were used to determine kinetic parameters. Variation of acetoacetate concentration (0.2–1 mM) at a series of fixed NADH concentrations (14–84 M) leads to a family of straight-line plots which intersect to the left of the $1/v$ axis and above the $1/S$ axis. When the data are plotted as a variation in NADH concentration at fixed acetoacetate levels, a similar plot is obtained. When the same experiments were carried out at pH 8, intersecting lines were obtained. At both pH values, substrate inhibition by NADH was observed at concentrations above 0.1 mM. However, these lines intersect close to the $1/S$ axis. Kinetic parameters were obtained from replots derived from the plots above.[14] These parameters are listed in Table I.

Inhibition of reactions by the presence of products gives important kinetic information.[15] Summaries of the inhibition patterns of the various substrates and products (including coenzymes) with respect to one another are summarized in Table II. These patterns are consistent with a reaction scheme in which ketone or alcohol can bind only if the appropriate

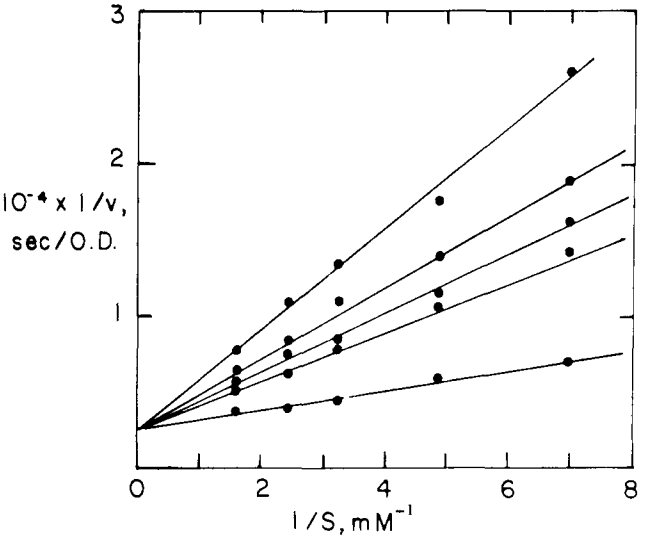

Figure 2. Initial rate of reduction of acetoacetate (S) at 37 °C, 0.1 M potassium phosphate. Concentrations: HBDH, 6.8×10^{-3} unit/mL; NADH, 1.58×10^{-4} M; MACP × 10^3 (bottom to top), 0.0, 1.72, 2.59, 3.45, and 5.17 M.

coenzyme has been bound first. This is because it can be seen that the coenzymes are able to compete directly against one another for the enzyme in order to be competitive. The substrates are noncompetitive, which is a kinetic pattern that results from the two materials binding to different enzyme forms, that is, enzymes to which the coenzymes have bound.[15]

Inhibitors of Reduction of Acetoacetate. It has been reported that the oxidation of D-3-hydroxybutyrate by HBDH-NAD is competitively inhibited by a variety of anionic materials.[4] Thus, it could be expected that these materials would also serve as noncompetitive inhibitors for the reduction of acetoacetate as product analogues. However, inhibitors of acetoacetate reduction that combine with the acetoacetate binding site of HBDH-NADH are limited to one reported case with enzyme from another source.[16] The inhibitor, 2-epoxypropionate, did not give competitive inhibition with the *P. lemoignei* enzyme or, in our hands, with the reported *Rhodopseudomonas spheroides* enzyme. Since we are interested in the sources of specificity with respect to acetoacetate, we sought particularly to define requirements of inhibitors as well as substrates.

Phosphates, phosphonates, and carboxylates, as salts and as esters, were tested for their ability to cause inhibition of reduction of acetoacetate. Table III summarizes these results. The general effect of ionic strength was also determined. Increasing buffer concentration to 0.4 M decreased the observed catalytic rate by 16%. Addition of 0.6 M potassium chloride (equivalent ionic strength to additional buffer) to 0.1 M potassium phosphate buffer reduced the observed rate by 52%. Therefore, there is a general ionic strength effect.

The effect of ethylenediaminetetraacetic acid (EDTA) was also determined, since it had been reported that the activity of the *R. spheroides* enzyme decreases with time with EDTA, giving evidence for possible involvement of a metal ion.[17] At pH 7, 37 °C, in 0.1 M phosphate buffer, 6.25 mM EDTA reduced the enzymic reaction rate by 27%. This inhibition was not time dependent (same inhibition at 1, 15, 30, 45, 75, and 105 min), contrasting with the reported effect of EDTA on the *R. spheroides* enzyme.[17] The effect is comparable to that of other carboxylate anions and does not give evidence for a metal ion's involvement in the *P. lemoignei* enzyme.

Competitive Inhibitors. Compounds that were found to inhibit reactions (Table III) were tested for their mode of inhibition. As we have noted, it was known that many anions are competitive with respect to hydroxybutyrate.[4] This was also

Table II. Product Inhibition Patterns for the Reduction of Acetoacetate at 37 °C in 0.1 M Potassium Phosphate Buffer

inhibitor	variable substrate	fixed substrate (mM)	inhibn pattern[a]	k_{ii},[b] mM	k_{is},[b] mM
NAD	NADH	acetoacetate (0.30)	C	0.89	0.98
NAD	acetoacetate	NADH (0.071)	NC	3.30	1.94
3-hydroxybutyrate	NADH	acetoacetate (saturation)	UC		
3-hydroxybutyrate	acetoacetate	NADH (0.064)	NC	10.33	16.47

[a] C, NC, and UC denote competitive, noncompetitive, and uncompetitive inhibition, respectively. [b] k_{is} and k_{ii} represent inhibition constants from slope and intercept replots, respectively, according to ref 21.

Table III. Phosphates, Phosphonates, and Carboxylates as Inhibitors of HBDH-Catalyzed Reduction of Acetoacetate at 37 °C in 0.1 M Potassium Phosphate Buffer

compound	pH	concn, mM	% inhibn[a]
sodium acetonylphosphonate	5.9	3.3	16
	7.0	3.3	21
sodium monomethyl acetonylphosphonate	5.9	3.3	54
	7.0	3.3	43
dimethyl acetonylphosphonate	7.0	5.9	0
methanephosphonic acid	7.0	3.3	11
	7.0	16.7	24
methyl methylphosphonate	7.0	3.3	13
	7.0	16.7	22
dimethyl methylphosphonate	7.0	16.7	0
methyl phosphate	7.0	16.7	30
dimethyl phosphate	7.0	16.7	12
acetylphosphonate	7.0	3.3	10
	7.0	16.7	28
sodium monomethyl acetylphosphonate	7.0	16.7	60
dimethyl acetylphosphonate	7.0	16.7	0
pyruvate	7.0	16.7	22
oxalate	7.0	16.7	9

[a] Percent inhibition denotes the decrease in activity of the enzyme in the presence of the inhibitor relative to the activity of the enzyme without the inhibitor.

the case with most of the anions we tested (Table IV). However, the 3-ketophosphonate monoester, methyl acetonylphosphonate (MACP), was unique in being competitive against acetoacetate at pH 7. Figure 2 shows these results. Values for K_i were determined with the aid of a Dixon plot.[18] At pH 8 this compound gave noncompetitive inhibition patterns. Since neither the substrate nor the inhibitor can ionize further, this effect is likely to be due to the ionization of a group on the enzyme.

Alternative Substrates. A number of ketones were tested as possible substrates in order to determine the specificity of the enzyme toward nonmetabolic species. The following did not react (37 °C, 0.1 M phosphate, pH 7.0; all salts are sodium salts): acetonylphosphonate (3.3 mM), methyl acetonylphosphonate (3.3 mM), dimethyl acetonylphosphonate (3.3 mM), ethyl acetoacetate (3.3 M), methyl acetonylphosphonate (16.7 mM), and pyruvate (16.7 mM). Sodium acetonylsulfonate and lithium 2-methylacetoacetate were reduced enzymatically at pH 7 and are therefore substrates. The two substrates (acetonylsulfonate, 1.5–5.5 mM; 2-methylacetoacetate, 3.3–8.5 mM) gave Lineweaver-Burk plots with different NADH levels which are similar to that produced by acetoac-

Table IV. Inhibition Patterns for Inhibitors vs. Substrates at 37 °C in 0.1 M Potassium Phosphate Buffer

inhibitor	pH	inhibition pattern[a] for substrates: acetoacetate	NADH	3-hydroxybutyrate	K_i, mM
monomethyl acetonylphosphonate $CH_3COCH_2PO_2(OCH_3)^-Na^+$	7	C			1.65
pyruvate $CH_3COCO_2^-Na^+$	8	NC	UC		4.0
	7	U	U		
				C	6.40
acetonylphosphonate $CH_3COCH_2PO_2(OH)^-Na^+$	7			C	0.30
	8			C	3.94
monomethyl acetylphosphate $CH_3COPO_2(OCH_3)^-Na^+$	7			C	5.40
dimethyl phosphate $(H_3CO)_2PO_2^-$	7			C	8.50
methyl methylphosphonate $CH_3PO_2(OCH_3)^-$	7			C	7.30

[a] C, NC, and UC refer to competitive, noncompetitive, and uncompetitive inhibition, respectively.

Table V. Kinetic Parameters for the HBDH-Catalyzed Reduction of Alternative Substrates at 37 °C in 0.1 M Potassium Phosphate Buffer at pH 7.0

substrate A	substrate B	10^5k_a, M	10^4k_{ia}, M	10^3k_b, M	10^7V, ms^{-1}
NADH	acetonylsulfonate	2.67	0.73	59.5	1.53
NADH	2-methylacetoacetate	0.92	0.73	3.2	1.26

etate in general appearance. The kinetic data are summarized in Table V.

Kinetic Pattern. The results of initial velocity studies and inhibition patterns indicate that HBDH from *P. lemoignei* conforms to the kinetic pattern of related dehydrogenase enzymes.[19,20] That is, the kinetics are consistent with ordered binding (NADH precedes acetoacetate; hydroxybutyrate leaves prior to NAD). This pattern conforms to equations summarized by Cleland.[21]

Product inhibition patterns, in the nomenclature used by Cleland[21] for a sequential bireactant system, are summarized in Table II. The experimental results are consistent with the "ordered" mechanism and are incompatible with random binding. Distinction between the "Theorell-Chance" and "ordered Bi-Bi" mechanisms can be made by product inhibition studies.[15] These suggest that the ordered Bi-Bi mechanism occurs. However, since the Theorell-Chance mechanism requires that no ternary complex formation takes place, it is chemically unlikely that such a distinction is really necessary. Segel has pointed out that no case of a true Theorell-Chance mechanism (which is a limiting case of an ordered Bi-Bi mechanism) has actually been observed or is likely ever to be observed.[15]

Further evidence for the mechanistic assignment comes from the competitive inhibition results. Since MACP is competitive with respect to acetoacetate at pH 7 and uncompetitive with respect to NADH, NADH is likely to bind before acetoacetate (unless an exotic mechanism is to be invoked). If acetoacetate is added before NADH, we would expect MACP to be a noncompetitive inhibitor with respect to NADH. Furthermore, for the different substrates, the dissociation constants of the enzyme-NADH complexes, K_{ia}, are invariant, although the K_m values for the substrates vary considerably. This indicates that binding of NADH occurs without influence from the second substrate.

Kinetics and pH Effects. The optimal pH for reduction of acetoacetate is between 6.0 and 6.5 (for oxidation of hydroxybutyrate it is around 8.0[4]). This is similar to that of HBDH from other sources.[17] Activity decrease results from an increase in K_m of both acetoacetate and NADH (Table I). This suggests that a cationic center may be involved in binding of the substrate.

Discussion

Our results indicate that HBDH to which NADH has bound will catalyze the reduction of 2-methylacetoacetate and acetonylsulfonate in addition to the metabolic substrate, acetoacetate. The finding of Delafield et al.[4] that homologues of acetoacetate are reduced, coupled with our observation concerning 2-methylacetoacetate, indicates that the active site is not sterically constricted. The increase in K_m for the larger substrates indicates that there are some unfavorable interactions, however. The fact that a 3-ketophosphonate monoester is a competitive inhibitor indicates that the catalytic apparatus makes a further distinction than does the binding apparatus. Binding appears to be satisfied by a compound with a carbonyl group three bond lengths from an oxyanionic center. Reduction of the carbonyl group is controlled by additional types of interactions.

Since both acetoacetate and hydroxybutyrate are anionic, it is likely that the same cation is responsible for electrostatic recognition, whatever coenzyme form binds initially. The general inhibition by monoanions supports this interpretation. The essential distinction made for binding appears to be recognition of the 3-keto group (at pH 7) after NADH has bound. The decrease in activity and change in the nature of inhibition by methyl acetonylphosphonate at pH 8 suggests that a group involved in this recognition undergoes an unproductive ionization with increasing basicity of the medium. This suggests that hydrogen bonding of a weak acid on the enzyme, such as the histidinium group proposed by Bloxham et al.[16] for lactate dehydrogenase and for HBDH from *R. spheroides*, is involved in this recognition. It has been suggested in general for NADH-dependent enzymic reductions which do not involve metal ions that a hydrogen-bond donor could act as a general-acid catalyst for hydride transfer.[22,23]

The abundance of competitive inhibitors for the oxidation of D-3-hydroxybutyrate catalyzed by HBDH indicates that the enzyme form to which NAD has bound is not as restrictive as is that to which NADH has bound. The site to which D-3-hydroxybutyrate binds does recognize the electrostatic requirement of the anionic substrate, but it appears that no specific recognition of the hydroxyl function occurs. The proposal that the carbonyl group of acetoacetate is hydrogen bonded at the active site of HBDH can account for this contrast. A hydrogen-bond donor, involved in catalysis of reduction of the carbonyl group, should function as a precursor to general-acid catalysis of reduction. Approaching the transition state from the other direction, the conjugate base of the donor should be involved in oxidation of the alcohol, functioning as a general base. Since attaining the transition state for the hydride-transfer reaction involves the greater energetic barrier of breaking the C-H bond, transfer of the proton of the hydroxyl group to a base would occur to only a small extent in the transition of the catalytic step. This is mirrored in the direction of reduction of the substrate. Avoidance of formation of the

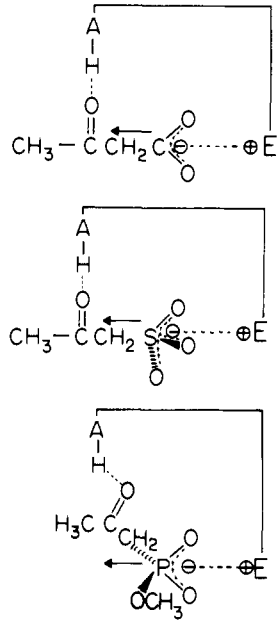

Figure 3. Electrostatically defined binding causes a phosphonate monoanion to be skewed compared to a sulfonate or carboxylate due to differences in symmetry of charged centers. Effect on the proposed hydrogen bond is illustrated.

alkoxide ion[24] of the reduced product (an energetically unfavorable species in neutral solution) would lead to a transition state in which the proton is far from the weak conjugate base of the hydrogen-bond donor.

The fact that a 3-ketophosphonate is not reduced, while 3-keto carboxylates and a 3-ketosulfonate are reduced, gives further information. We have suggested that there are specific interactions of a cationic binding site with the anionic portion of the substrate and a hydrogen-bonding interaction of the carbonyl group with an acidic group. For the hydrogen-bonding interaction, both specificity and catalysis are likely to be involved.

Figure 3 illustrates differences and similarities in binding of these classes of compounds when two centers are involved. We assume that a defined electrostatic interaction between the anionic substrate and a cationic center on the enzyme is responsible for initial recognition. The electrostatic vector of the functional group then is aligned with respect to the cationic center. The resultant effect is that the remainder of the substrate is constrained to a portion of the enzyme's active site defined by the electrostatic interaction.

For carboxylate substrates, the resultant vector coincides with its C_1–C_2 bond since the carboxylate functionality is rotationally symmetric. The sulfonate group contains a similar resultant vector since its functional group is also rotationally symmetrical. The phosphonate monoanion is not rotationally symmetric. The resultant vector of this group is skewed with respect to the C_2–P bond. Therefore, the 3-ketophosphonate will hydrogen bond at a different angle than do 3-ketosulfonates or 3-keto carboxylates which are rotationally symmetric at their anionic center. The hydrogen-bonding angle difference does not alter specifity with respect to binding but it does alter catalytic specificity since the relationship to the coenzyme must also become distorted.

It has been shown that the angular dependence of hydrogen bonding is not steep[25,26] and minima are not sharp. This would allow favorable interactions to occur even with a rather poorly aligned substrate. We have suggested that in the direction of reduction of substrate, proton transfer from the catalytic acid to the carbonyl group of the substrate is nearly complete in the transition state as is hydride transfer from the coenzyme. That is, the transition state resembles the substrate in its reduced form and the catalytic group in its conjugate base form. Distortion of the hydrogen-bonding interaction does not produce a high-energy situation with respect to binding, but this does block hydride transfer, as evidenced by the failure of the ketophosphonate to be a substrate. Product formation is certain to be more sterically restricted than is binding (in either direction). A process involving formation of two covalent bonds will be contrained to a steeper trough than will maintenance of a single hydrogen bond, since the covalent bonds have higher bending and stretching force constants.[25,27] The coenzyme and carbonyl group of the ketophosphonate are then unable to react because correct alignment and proximity become impossible to achieve.

References and Notes

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