H_2SO_4 at the same temperature. Essentially, the same result was reported on the resin-catalyzed hydrolysis of *n*-butyl acetate.[28]

In summary, the accumulation of substrate molecules inside the polymer phase contributes to the resin additional catalytic power over aqueous hydrogen ions. The values given in Tables III and VI show that the rigid resin matrix takes advantage unefficiently from the forces involved in the transfer of hydrophobic substrates. The data support the correlation suggested above between catalytic power and catalyst molecular flexibility.

Kinetic Solvation Pressure in Enzyme Reactions. The catalytic power of enzymes can be accounted for in terms of two fundamental contributions: (1) a negative change in the solvation pressure of substrate molecules when transferred from the bulk of the medium to the active site, plus (2) the stabilization of the TS complex by the catalyst's microenvironment.

The first term can be obtained as $+RT(\partial \ln K_s/\partial \bar{V})_T$, where K_s is the enzyme–substrate complex dissociation constant. For many cases, $K_m = K_s$ and this contribution is therefore approximately equal to $+RT(\partial \ln K_m/\partial \bar{V})_T$.

The second contribution is $-RT(\partial \ln k_{cat}/\partial \bar{V})_T$. The model systems discussed above failed to give any important contribution to the second effect; most of its catalytic power over aqueous H^+ came from a modestly favorable partition of substrate molecules from the bulk aqueous medium to the catalytic pseudophase.

Contrary to the crude model systems, chymotrypsin can successfully stabilize the TS complex for the hydrolysis of a family fo AcNHCHRCO$_2$Me esters.[35] The substrate binding contribution to the KSP is -182 J cm^{-3}, and the further TS complex stabilization by increased hydrophobic interaction leads to a contribution to KSP of -130 ± 10 J cm^{-3}.

Another interesting case is that of lipolytic enzymes that perform their catalytic actions at oil–water interfaces,[32] such as pancreatic lipase and horse liver esterase. These enzymes catalyze the hydrolysis of carboxylic esters when the latter are located at the hydrophobic interfaces of microemulsions. Thus, the initial state of the substrate is that of two-dimensional freedom at the interface.

The overall KSP for the hydrolysis of small esters by pancreatic lipase or horse liver esterase can thus be dissected into three contributions: (1) a value ΔSP(1) for the transfer of reactant-state nonpolar moieties (RS-X) from hydrophobic interfaces to bulk water medium, (2) a value ΔSP(2) for the transfer of RS-X's from bulk water to the hydrophobic catalytic sites of the enzymes, and (3) the actual amount for stabilization of the corresponding TS complexes, ΔSP(3).

(RS-X, interface) → (RS-X, bulk H_2O)

(RS-X, bulk H_2O) → (RS-X, enzyme)

(RS-X, enzyme) → (TS-X, enzyme)

(RS-X, interface) → (TS-X, enzyme)

ΔSP(1) can be considered equal to the opposite of the change in solvation pressure for the transfer of nonpolar molecules from water to micelles, +120 J cm^{-3}; ΔSP(2) can be reasonably equated to the -200 J cm^{-3} reference figure for water/hydrocarbon extraction. This would require ΔSP(3) = -130 J cm^{-3} to make an overall KSP of -210 J cm^{-3}, a figure similar to the KSP observed for triacylglycerol hydrolysis catalyzed by castor bean acid lipase under saturation conditions.[36] (-136 J cm^{-3}). The hydrophobic contribution of the catalytic power of resins, micelles, and polyions makes them interesting partial models for enzymic reactions, because the supramolecular arrays can also produce rate accelerations by bringing the reacting molecules together, by use of weak binding forces, in much the same way an enzyme uses a part of the intrinsic binding energy of the substrate (in the form of its TS complex) to pay for the entropy cost of assembling the reactive complex.[31] The rigid nature of the resin makes this repayment process less effective than in more flexible catalysts, as evidence by the magnitude of the KSP values.

A further characteristic of enzymes is that water molecules present within active sites in the reactant state can be displaced from the active site upon TS complex formation. Thus, it is appropriate to say that the catalytic efficiency should increase with the molecular flexibility of catalytic sites.

These results suggest that the natural evolutionary pathway in enzyme design is to produce flexible active-site structures, which take full advantage of the noncovalent binding forces developed in the TS.

Acknowledgment. I thank Profs. M. M. Kreevoy, E. Grundwald, and S. Lindenbaum for their interest and helpful comments. This paper was completed during the tenure of a Fulbright Fellowship at the Kansas University Chemistry Department.

Thiamin Diphosphate Catalysis. Mechanistic Divergence as a Probe of Substrate Activation of Pyruvate Decarboxylase

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Abstract: Pyruvate decarboxylase is a thiamin diphosphate dependent enzyme that catalyzes the conversion of pyruvate to acetaldehyde and carbon dioxide. The substrate activates the enzyme, with kinetic patterns indicating a cooperative effect between two binding sites (Hill coefficient, 1.5). An alternative substrate, 3-fluoropyruvate, is converted to acetate, fluoride, and carbon dioxide. This reaction is not subject to activation, displaying normal Michaelis–Menten kinetics, but 3-fluoropyruvate activates the enzymic reaction of pyruvate. The dual reaction pattern was used as a probe of the cooperative phenomenon. Inhibition patterns show that 3-fluoropyruvate interacts with the enzyme at the same site as does pyruvate and that the affinities are similar. Since the reaction of 3-fluoropyruvate proceeds through a mechanism paralleling that of pyruvate up to the step in which carbon dioxide is lost, the step in the mechanism that is regulated occurs after the point at which the mechanisms diverge. The reaction of 3-fluoropyruvate produces enzyme-bound 2-(1-acetyl)thiamin diphosphate, which is readily hydrolyzed to produce acetate and holoenzyme, while the reaction of pyruvate produces the much less reactive enzyme-bound species 2-(1-hydroxyethyl)thiamin, which undergoes elimination of acetaldehyde to produce holoenzyme. These results suggest that the conversion of the complex of enzyme and 2-(1-hydroxyethyl)thiamin diphosphate to acetaldehyde and the holoenzyme is subject to allosteric control.

The thiamin diphosphate enzyme pyruvate decarboxylase is subject to activation by its substrate,[1-3] but the mode by which this activation occurs is unknown. The detailed mechanism for the catalytic process of the enzyme was proposed by Breslow to

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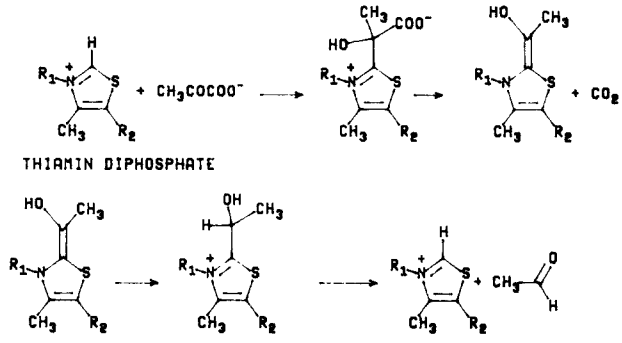
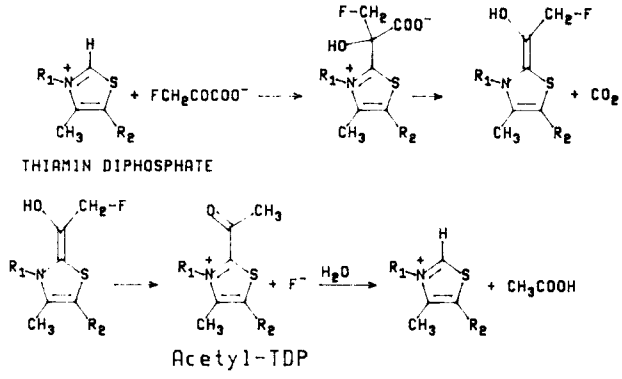
Scheme I



Scheme II



involve a series of covalent intermediates derived from the substrate and the 2-position of the coenzyme: 2-(2-lactyl)thiamin diphosphate from which carbon dioxide is lost to produce 2-(1-hydroxyethyl)thiamin diphosphate, the precursor of acetaldehyde (Scheme I).[4] The properties of these intermediates have been established.[5] The relationship between the regulatory process and the steps in the catalytic mechanisms is unknown.

Schellenberger and co-workers have shown that the activation of pyruvate decarboxylase by its substrate occurs at an allosteric site and that pyruvamide, which is not a substrate, will also function as an activator.[6,7] Alvarez and Schowen analyzed isotope effects on the steady-state kinetic parameters of the enzyme and suggested that the carbonyl group of the activating molecule reacts reversibly with a nucleophilic group of the enzyme.[8]

Leung and Frey observed that another thiamin diphosphate dependent enzyme, bacterial pyruvate dehydrogenase, catalyzes the conversion of 3-fluoropyruvate to acetate, fluoride, and carbon dioxide.[9] They proposed that the conversion involves loss of carbon dioxide and fluoride from the adduct of 3-fluoropyruvate and thiamin diphosphate, producing 2-(1-acetyl)thiamin diphosphate which undergoes hydrolysis (Scheme II). The mechanism of this reaction diverges from that of pyruvate at the step in which carbon dioxide is released. If a similar reaction occurs with 3-fluoropyruvate and pyruvate decarboxylase, this alternative reaction can provide a useful probe for determining the relationship between the catalytic mechanism and the regulatory process. Rate enhancement necessarily requires a reduction in the barrier to the rate-determining step. The response of divergent reactions to the activation process should specify whether regulation occurs before or after the point of divergence.

In this paper we report that pyruvate decarboxylase catalyzes the conversion of 3-fluoropyruvate to acetate, fluoride, and carbon dioxide. Unlike the reaction of pyruvate, the process is not subject to activation but 3-fluoropyruvate activates the reaction of pyruvate. The contrast indicates the mechanistic basis for the regulatory divergence.

Experimental Section

Materials. Sodium 3-fluoropyruvate, sodium pyruvate, NADH, thiamin diphosphate, and yeast alcohol dehydrogenase were purchased from Sigma Chemical Co. Buffers and solutions of inorganic materials were prepared from Fisher reagent chemicals. Standardized buffers were used for all pH measurements.

Pyruvate decarboxylase was isolated from raw wheat germ following published procedures.[10] The enzyme has been isolated from brewer's yeast as well as from wheat germ, and recent studies have shown that the enzymes from the two sources are in most respects identical.[11] The solid that resulted from precipitation with a solution of saturated ammonium sulfate was dissolved in 1 mL of 100 mM imidazole buffer, pH 6.8 at 5 °C. Undissolved material was removed by centrifugation (15 000 rpm, Sorvall RC2B, SS-34 rotor) at 5 °C for 5 min. The supernatant liquid, diluted with 100 mM pH 6.0 sodium phosphate containing 20 mM magnesium sulfate and 25 μM thiamin diphosphate, was used for kinetic studies.

Methods. The temperature of reaction solutions was maintained at 30 °C in a jacketed beaker or the jacketed sample holder of a spectrometer through which water was circulated. The circulating bath was regulated by a Versa-Therm proportional electronic temperature controller, Model 2156, with a Yellow Springs 700 thermistor probe. Solutions were prepared immediately before each set of experiments.

The activity of pyruvate decarboxylase was assayed by three independent methods: (1) The production of acetaldehyde due to the enzyme-catalyzed decarboxylation of pyruvate was monitored by a coupled assay in which it is reduced with NADH and yeast alcohol dehydrogenase.[10] The decrease in absorbance at 340 nm accompanying the oxidation of NADH was recorded (Unicam SP-1800 spectrometer). (2) The production of fluoride ion from the reaction of 3-fluoropyruvate was monitored with a fluoride ion specific electrode (Corning) vs a standard calomel electrode on a Radiometer PHM28 meter equipped with a Heath SR-205 recorder. Potassium fluoride (80 μM) was added to provide a stable background for the measurement of fluoride. (3) Carbon dioxide production was monitored by use of an Orion carbon dioxide electrode in place of the fluoride electrode with the equipment used for the fluoride electrode.[12] The electrode systems were calibrated over the range of concentration measurements before each experiment.

Results

Wheat germ pyruvate decarboxylase catalyzes the conversion of 3-fluoropyruvate exclusively to fluoride, acetate, and carbon dioxide, the same products Leung and Frey observed when this substrate reacted with pyruvate dehydrogenase.[9] If the reaction of this substrate with wheat germ pyruvate decarboxylase completely paralleled that of pyruvate, decarboxylation of the enzymic adduct of 3-fluoropyruvate and thiamin diphosphate followed by protonation would generate enzyme-bound 2-(2-fluoro-1-hydroxyethyl)thiamin diphosphate. The enzyme would catalyze the release of 2-fluoroacetaldehyde from the adduct. A test using alcohol dehydrogenase and NADH to detect the production of 2-fluoroacetaldehyde (a known substrate of alcohol dehydrogenase)[13] was negative.

The enzyme-catalyzed reaction of 3-fluoropyruvate requires thiamin diphosphate as a cofactor, since enzyme that was extensively dialyzed failed to catalyze any reaction. Upon reconstitution of the holoenzyme with thiamin diphosphate, maximal activity was regained.

The rate of enzyme-catalyzed production of fluoride from 3-fluoropyruvate follows a zero-order profile and the lag phase, which is characteristic for the reaction with pyruvate,[7] is absent. As well, there is no sigmoidicity in the plots of velocity vs substrate

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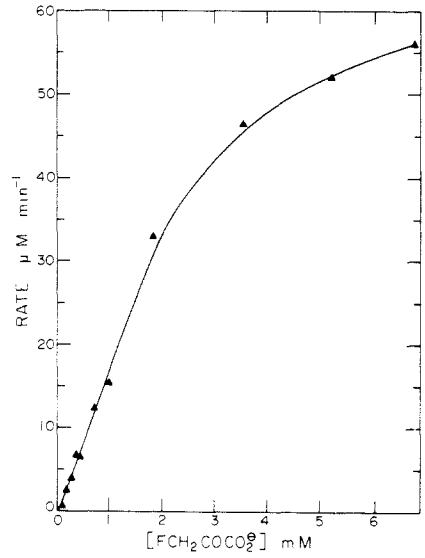

Figure 1. Kinetic profile of the wheat germ pyruvate decarboxylase catalyzed release of fluoride from 3-fluoropyruvate at low substrate concentration. Fluoride release was measured with a fluoride ion specific electrode for solutions (25-mL total volume) containing 100 mM sodium phosphate buffer (pH 6.0), potassium fluoride (80 μM), 3-fluoropyruvate, and wheat germ pyruvate decarboxylase. The reaction was initiated by addition of enzyme.

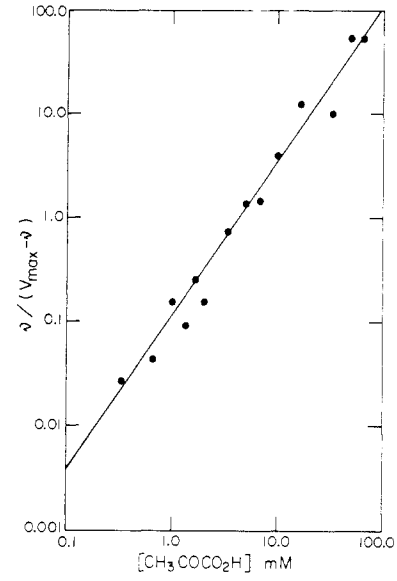

Figure 2. Hill plot of the reaction of pyruvate catalyzed by wheat germ pyruvate decarboxylase. The decrease in absorbance at 340 nm was measured for 3.0-mL solutions containing 100 mM sodium phosphate (pH 6), 3.3 μM NADH, 0.55 mg of yeast alcohol dehydrogenase, sodium pyruvate, and wheat germ pyruvate decarboxylase. The reaction was initiated by addition of the enzyme.

concentration. In particular, reactions of low concentrations of 3-fluoropyruvate were examined where sigmoidicity would be most apparent (Figure 1). On the basis of these observations, it is clear that there is no substrate activation of the 3-fluoropyruvate reaction. As a confirmatory test, the release of carbon dioxide was monitored under the same conditions (data not presented) and similar results were obtained. The V_{max} and K_m (4.2 mM) for release of carbon dioxide are the same as those obtained when the rate of release of fluoride was determined. Under the same conditions it was found that, using this assay, the V_{max} for pyruvate is 3 times that for fluoropyruvate.

In contrast, when the reaction of pyruvate with wheat germ pyruvate decarboxylase is measured by using the coupled assay with alcohol dehydrogenase, there is a considerable lag phase. It is accentuated at low concentrations of pyruvate. Analysis of the data shows that the enzyme has an affinity ($S_{0.5}$) for pyruvate of ~2.2 mM and a Hill coefficient[14] of 1.5 (Figure 2). The magnitude of the Hill coefficient indicates the presence of positive cooperativity. For comparison, a plot of the data for 3-fluoropyruvate gives a coefficient of 1.0, indicating that this substrate's reaction is not subject to activation. These results are consistent with the hypothesis of Schellenberger and Huebner[7] for yeast pyruvate decarboxylase: There are two binding sites with equal affinity for pyruvate. One of the sites is catalytic and the other is regulatory. There are two likely explanations for the nonactivating kinetic behavior of the enzyme with 3-fluoropyruvate: (1) 3-Fluoropyruvate binds exceptionally tightly to the regulatory site so that the enzyme is always fully activated, or (2) the conversion of 3-fluoropyruvate is not subject to allosteric regulation. These modes were distinguished by testing whether 3-fluoropyruvate activates the reaction of pyruvate. If 3-fluoropyruvate binds tightly to a regulatory site, then it should activate the reaction of either substrate.

Since the products of the reaction of 3-fluoropyruvate with the enzyme do not interfere with the coupled assay used to detect the production of acetaldehyde from pyruvate, the effect of 3-fluoropyruvate on the pyruvate reaction is readily determined. In Figure 3 it is shown that high concentrations of 3-fluoropyruvate will competitively inhibit the conversion of pyruvate to acetaldehyde with K_i = 3.0 mM. This result is consistent with both compounds interacting with thiamin diphosphate at the catalytic

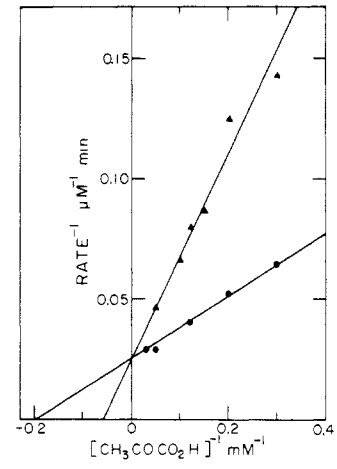

Figure 3. Inhibition by 3-fluoropyruvate of the reaction of pyruvate catalyzed by wheat germ pyruvate decarboxylase. The decrease in absorbance at 340 nm was measured for 3.0-mL solutions containing 100 mM sodium phosphate (pH 6), 3.3 μM NADH, 0.55 mg of yeast alcohol dehydrogenase, wheat germ pyruvate decarboxylase, sodium pyruvate, and sodium 3-fluoropyruvate; (●) 0 mM, (▲) 20 mM.

site. (A complementary experiment showed that pyruvate competitively inhibits the release of fluoride from 3-fluoropyruvate; K_i = 2.3 mM.)

If the affinity of 3-fluoropyruvate for the regulatory site is higher than its affinity for the catalytic site, activation of the pyruvate reactions would necessarily be accomplished by low concentrations of 3-fluoropyruvate. This is not the case. In addition, an estimate of the affinity can be made from observing the concentration of 3-fluoropyruvate necessary for full activation of the enzyme. If the binding were extremely tight, then very low concentrations would give full activation. Figure 4 shows that 3-fluoropyruvate does bind to the regulatory site, activating the pyruvate reaction, but the concentrations necessary for the activation indicate that the affinity is not higher than that of pyruvate.

Discussion

Wheat germ pyruvate decarboxylase displays different kinetic properties in the reactions it promotes with pyruvate and the alternative substrate 3-fluoropyruvate. In the pyruvate reaction,

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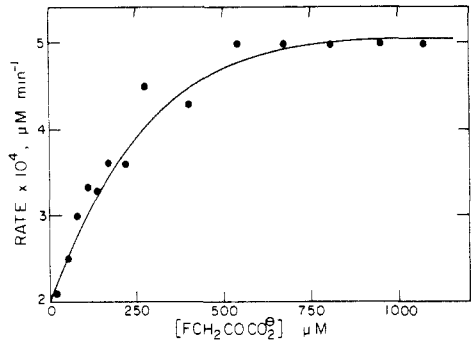

Figure 4. Activation of the reaction of pyruvate with wheat germ pyruvate decarboxylase by 3-fluoropyruvate. The decrease in absorbance at 340 nm was measured for 2.7-mL solutions containing 100 mM sodium phosphate (pH 6), 3.3 μM NADH, 0.55 mg of yeast alcohol dehydrogenase, wheat germ pyruvate decarboxylase, 280 μM sodium pyruvate, and sodium 3-fluoropyruvate. The reaction was initiated by addition of enzyme.

sigmoidal rather than normal hyperbolic kinetic behavior is observed. This indicates that there are interactions between at least two pyruvate binding sites (which may differ in structure). In contrast, we have shown that the reaction of 3-fluoropyruvate displays normal hyperbolic kinetics. The lack of activation in the 3-fluoropyruvate reaction is not due to tight binding of this material by the enzyme. The activation of the pyruvate reaction by 3-fluoropyruvate requires concentrations on the order of $S_{0.5}$ of pyruvate. Furthermore, since at these concentrations (0.5 mM) 3-fluoropyruvate also reacts as a substrate, the observed maximal rate for pyruvate decarboxylation does not represent maximal activation by 3-fluoropyruvate but a balance between activation at a regulatory site and inhibition at a catalytic site.

The contrasting behavior of the enzyme with the two substrates serves to indicate the source of the activation process. The differences between the two reaction processes localize the steps in the pyruvate reaction that are affected by the cooperative interaction between sites. The mechanism for the decarboxylation of pyruvate is shown in Scheme I. The conversion of 3-fluoropyruvate should follow the mechanism in Scheme II, in analogy to that proposed for reaction of 3-fluoropyruvate with pyruvate dehydrogenase by Leung and Frey.[9] The two substrates undergo reaction by a common set of steps, but these diverge after carbon dioxide is lost from the adduct of the substrate with thiamin diphosphate. The enzyme-bound adduct of pyruvate and thiamin diphosphate extrudes carbon dioxide and then is protonated to yield 2-(1-hydroxyethyl)thiamin diphosphate, which in turn is converted to acetaldehyde and enzyme-bound thiamin diphosphate.[15] The reaction of 3-fluoropyruvate forms an adduct of thiamin diphosphate, which loses carbon dioxide and fluoride ion to generate 2-(1-acetyl)thiamin diphosphate (Acetyl-TDP in Scheme II). 2-(1-Acetyl)thiamin diphosphate hydrolyzes to yield acetate and enzyme-bound thiamin diphosphate. The mechanisms diverge after the loss of carbon dioxide from the adduct of the substrate with thiamin diphosphate.

The point of divergence of the mechanisms indicates that the activation of the pyruvate reaction occurs in a step following the loss of carbon dioxide from enzyme-bound lactylthiamin diphosphate: either elimination of acetaldehyde from 2-(1-hydroxyethyl)thiamin diphosphate or release of acetaldehyde and regeneration of the free holoenzyme. The release of acetaldehyde from yeast pyruvate decarboxylase is known to be rate-determining,[16] and control can be effective at this step.

The hydrolysis of enzyme-bound 2-(1-acetyl)thiamin diphosphate (Scheme II) is not part of the enzyme's normal catalytic operation. Therefore, either the spontaneous rate of the hydrolysis is sufficient to permit the enzyme-catalyzed processes to proceed or the catalytic apparatus of the enzyme is "recruited" for the hydrolytic activity. The hydrolysis of 3,4-dimethyl-2-(1-acetyl)thiazolium chloride has been studied by Lienhard as a model for the hydrolysis reactions of 2-(acetyl)thiamin diphosphate.[16] The compound rapidly forms a covalent hydrate, which then undergoes an elimination reaction to generate acetate and 3,4-dimethylthiazolium chloride. The reaction is slow in acidic solutions and the acetyl derivative has a unique absorption, leading Lienhard to suggest that the enzymatic intermediate might be trapped and identified in acidic solution. Recently, Frey and co-workers prepared 2-(1-acetyl)thiamin diphosphate.[17] The material has hydrolytic reactivity similar to that of Lienhard's model compound. Effects of the pyrimidine ring significantly modify the reactivity patterns with respect to nucleophiles other than water.

The rate constant for the uncatalyzed addition of water to 3,4-dimethyl-2-(1-acetyl)thiazolium ion to form the hydrate is 6×10^{-2} s^{-1} at 25 °C, while the second-order rate constant for the reaction with hydroxide is 1.5×10^6 M^{-1} s^{-1}.[16] At pH 7, the observed rate constant in the absence of buffer is 0.21 s^{-1}. The reaction is further subject to general-base catalysis by buffer species. The decomposition of the hydrate to expel the thiazolium ylide has an observed rate consatant of $\sim 1.7 \times 10^{-1}$ s^{-1} at pH 7, 25 °C. The expulsion of the thiazolium ylide from the hydrate of the acetylthiazolium compound is base-catalyzed, proceeding via the intermediate in which the hydroxyl group of the hydrate is converted to its conjugate base. For comparison, the rate constant for elimination of pyruvate from lactylthiamin is a reasonable model for the rate constant for the elimination of acetaldehyde from 2-(1-hydroxyethyl)thiamin. The rate constant for this process at pH 7, 25 °C, is 1.3×10^{-7} s^{-1},[18] which is less than 10^{-6} times the rate constant for loss of the acetyl group from 2-(1-acetyl)thiamin. If the rate-determining step of the enzyme is normally associated with the elimination of acetaldehyde, a different step will be rate-determining for the elimination of acetate from 2-(1-acetyl)thiamin diphosphate on the enzyme since this process is inherently much more rapid.

However, the elimination of acetate still should be catalyzed by the enzyme. The value of k_{cat} for (activated) pyruvate decarboxylase from yeast is 40 s^{-1} (pH 6, 30 °C).[19] We have observed the value of V_{max} for the reaction of 3-fluoropyruvate under these conditions and it is $^1/_3$ that of pyruvate; therefore, k_{cat} is 13 s^{-1}. Thus, the predicted spontaneous rate for the decomposition of 2-(1-acetyl)thiamin diphosphate is somewhat smaller than the overall enzymic rate of decarboxylation of pyruvate. A Brønsted base must be present at the active site of the unactivated enzyme to permit the elimination of acetaldehyde, since acetaldehyde is eliminated even if the enzyme is unactivated. The activation process, which would enhance the rate of elimination of acetaldehyde, would have no further effect on the hydrolysis of 2-(1-acetyl)thiamin diphosphate where another step would be rate-determining. The kinetics of decomposition of enzyme-bound synthetic 2-(1-acetyl)thiamin diphosphate[17] should provide an important test of the estimates. In contrast, the hydrolysis of 2-(1-benzoyl)thiamin diphosphate, generated by the reaction of (*p*-bromobenzoyl)formate with benzoylformate decarboxylase, appears to be rate-determining since a benzoylated derivative of thiamin diphosphate accumulates and slows the catalytic reaction.[20]

As noted earlier, isotope effects on the reaction catalyzed by pyruvate decarboxylase have indicated that a nucleophile on the enzyme may react reversibly with the effector.[8] The addition process could improve the orientation of a catalytic group involved in the formation of acetaldehyde from enzyme-bound 2-(1-hydroxyethyl)thiamin diphosphate. Finally, it should be noted that the activation process by 3-fluoropyruvate occurs at site that does not coincide with the catalytic site[8] and that the divergence

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after decarboxylation does not change the state of activation of the enzyme.

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Registry No. Pyruvate decarboxylase, 9001-04-1; pyruvate, 127-17-3; 3-fluoropyruvate, 433-48-7.

Effect of Heme Orientation on the Reduction Potential of Cytochrome b_5

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Abstract: Bovine erythrocyte cytochrome b_5, a soluble protein, and bovine microsomal cytochrome b_5, solubilized by trypsin cleavage, were isolated and purified, and their proton NMR spectra were compared. These two proteins had identical chemical shifts of the heme resonances and the same ratio of major to minor heme orientation. Thus, spectroelectrochemical titrations were carried out on the trypsin-cleaved microsomal protein. In its equilibrium ratio of 9:1 major to minor heme orientation, cytochrome b_5 gave a reduction potential of -1.9 ± 1.6 mV vs SHE at $\mu = 0.13$ M, pH 7.0, and $T = 24$ °C, utilizing three electrochemical mediators, 0.2 mM $Ru(NH_3)_6Cl_3$, 0.2 mM $K_3Fe(CN)_6$ and 1 mM methyl viologen. Spectroelectrochemical titrations of the apoprotein freshly reconstituted with hemin, in which the major:minor ratio was close to 1:1, yielded a reduction potential of -10.0 ± 1.7 mV vs SHE under the conditions listed above. The potentials of two samples of reconstituted cytochrome b_5 that had been allowed to equilibrate for 3 days were both -1.8 mV, indicating a return to the equilibrium ratio of major to minor forms. NMR quantitation of the time course of heme rotation in H_2O under the electrochemical conditions has led to an estimate of major:minor ratio of 60:40 at the midpoint of the titration and a half-life for heme reorientation of 12 ± 1 h. These data lead to calculated reduction potentials of $+0.8$ and -26.2 mV, respectively, for pure major and minor heme orientations. Although this difference is probably not large enough to be physiologically significant, the fact that the difference exists suggests that a detailed correlation between structure and reduction potential is very important for understanding the reduction potentials of heme proteins in general.

Cytochrome b_5 is a heme protein that exists in soluble form in erythrocytes and in membrane-bound form in liver microsomes. Erythrocyte cytochrome b_5 has been shown to mediate the reduction of methemoglobin by NADH-cytochrome b_5 reductase in normal red cells.[1-3] The presence of cytochrome b_5 in erythrocytes stimulates methemoglobin reduction by NADH-cytochrome b_5 reductase by as much as 77-fold.[1] The protein is thus important in reducing the ca. 3% of hemoglobin that is oxidized to the met form each day in normal humans.[4] Low levels of erythrocyte cytochrome b_5 may be associated with some forms of methemoglobinemia.[1]

Microsomal cytochrome b_5 is a membrane-bound amphiphatic redox protein that functions as a component of a microsomal electron-transfer chain in endoplasmic reticulum membranes. It has also been shown to be bound to outer mitochondrial membranes[5] and in plasma membranes from intestinal microvilli and erythrocytes[6] as well as in the mitochondrial intramembrane space.[7] There is evidence that it participates in the microsomal stearyl-CoA desaturation reaction.[8] It is also known to interact with cytochrome P450 as an alternate electron donor and to act as a respiratory carrier during hepatic microsomal mixed-function oxidation reactions.[9] It is a two-domain protein, with a hydrophilic, globular, heme-containing domain that is anchored to the membrane by a hydrophobic tail.[10] The membrane-bound protein can be solubilized by detergents or by treatment with trypsin, which cleaves the hydrophobic tail, as well as the first three amino acids of the N-terminus.[11,12]

The protein sequence of the two individual types of bovine erythrocyte cytochrome b_5 is the same as the first 95 or 97 (types I and II, respectively) amino acids of bovine microsomal b_5, indicating that the two proteins may be specified by the same gene. It has been suggested that the soluble erythrocyte protein is derived from the microsomal protein by proteolysis during erythroid maturation.[13] Trypsin cleavage of microsomal cytochrome b_5 produces a protein that consists of residues 3-86 of the original membrane-bound protein.[11,12] It thus differs from the type II erythrocyte protein in having 3 less amino acids at the N-terminal end and 11 less at the C-terminal end.

Trypsin-cleaved cytochrome b_5 has been the subject of several NMR spectroscopic investigations.[14-18] Ferricytochrome b_5

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