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Mini-review

On the importance of being zwitterionic: enzymatic catalysis of decarboxylation and deprotonation of cationic carbon☆

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Abstract

Carbanion ylides are strongly stabilized by electrostatic interactions between opposing charges at neighboring atoms and this stabilizing electrostatic interaction increases with decreasing dielectric constant of the medium through which the charges interact. Consequently, there is a large increase in the thermodynamic driving force, with decreasing dielectric constant of the reaction medium, for deprotonation of cationic carbon acids and decarboxylation to form related ylides. This favors catalysis of the formation of unstable ylides at enzyme active sites of low dielectric constant. A brief survey of enzymes that catalyze deprotonation of cationic carbon acids and related decarboxylation reactions shows catalysis generally occurs for substrates that are bound in a deep pocket on the protein, with an apparent dielectric constant that is much lower than for the solvent water. In several cases, proton transfer is to a catalytic residue that is relatively weakly solvated in water. We suggest that there is a strong advantage for evolution of protein catalysts that utilize weakly solvated basic side chains which are relatively easily buried in nonpolar active sites that are favorable for zwitterion formation.

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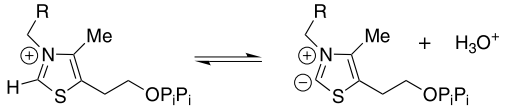
1. Introduction

It is generally assumed that enzyme catalysts make good use of those properties of proteins that favor stabilization of the transition state for the catalyzed reaction. This leads to an apparent dichotomy between the strong stabilization of ionic transition states for polar reactions in the solvent water, which provides strong solvation of free ions; and the stronger stabilization, relative to reactant, of formation of the same ionic transition states at enzyme active sites with effective dielectric constants that are by all accounts much lower than for water, and which might therefore be expected be to provide a less favorable environment for the formation of ions [1–5].

The observation of effective protein catalysis of heterolytic reactions at the relatively nonpolar milieu of enzyme active sites shows, not surprisingly, that proteins do more than simply provide a bulk medium for their catalyzed reactions. Enzyme active sites are littered with charged and polar groups that may assist in catalysis. These groups also have the effect of increasing the effective dielectric constant, but not to the value of water [1–4]. In addition, the magnitude of the stabilizing interaction between a charged transition state and an opposing charge or dipole is inversely proportional to the dielectric constant of the medium. Therefore, the stabilization of a transition state by a precisely oriented polar group of opposite charge or dipole moment at nonpolar enzyme active site will be stronger than the stabilization of the corresponding transition state in solution. This intramolecular electrostatic interaction will be favored entropically for an enzymatic reaction, compared with the development of the corresponding interaction for a bimolecular reaction in water [6].

The stabilization of zwitterions by interactions between opposing charges can be thought of as "internal" solvation, whose strength will increase with decreasing separation between charges until the limit is reached for ylides, where the charges lie at neighboring atoms. This stabilizing interaction strongly favors formation of ylides from deprotonation of cationic carbon, compared with formation of related carbanions from deprotonation of neutral carbon. This is reflected in the large carbon acidity of the thiazole ring of thiamine pyrophosphate (TPP, Scheme 1) [7] and for cationic ketones [8,9], esters and amino acid zwitterions [10–13]. It was suggested nearly 35-years ago that pyruvate decarboxylase catalyzes deprotonation of TPP, the first step in decarboxylation of pyruvate, by providing a medium of low dielectric constant which causes a shift in the equilibrium for proton transfer [14–16]. This prediction has since been substantially confirmed by experiment, as will be discussed in greater detail at the end of this paper.

There are a number of reactions that proceed through unstable zwitterionic carbanion intermediates (ylides). This review will highlight evidence obtained from studies on these enzymes that is consistent with the proposal that a substantial fraction of



Scheme 1.

their rate acceleration results directly from sequestration of substrate(s) at nonpolar active sites whose bulk dielectric constant is closer to the small value expected for a protein [1–4] than the value of $D = 79$ for water.

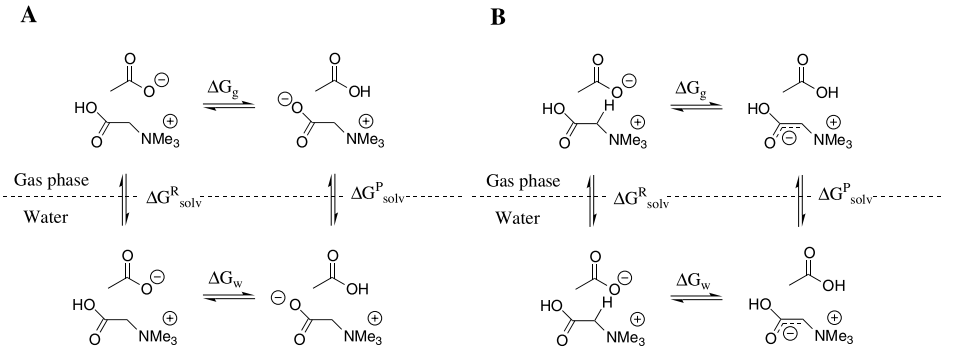
2. Amino acid racemases

2.1. Formation and stability of amino acid enolates in the gas phase and solution

There are several pyridoxal phosphate independent enzymes that catalyze racemization of amino acids through an enolate reaction intermediate, and sufficient data to allow a comparison between the thermodynamic driving force for carbon deprotonation of cationic amino acids to form enolate zwitterions in the gas phase and in solution [10,17].

The effect of a $-NMe_3^+$ substituent on the oxygen acidity of carboxylic acids shows an enormous dependence on the reaction medium (Scheme 2A) [18,19]. The addition of a $-NMe_3^+$ group to acetate ion results in only a small decrease in the basicity of the carboxylate group in water, $\Delta G_w = -4$ kcal/mol ($\Delta pK_a = 2.9$); but it results in a very large decrease in gas phase basicity, from 348.5 kcal/mol for $CH_3CO_2^-$ [20] to 239.2 kcal/mol [19] for $^+Me_3NCH_2CO_2^-$, $\Delta G_g = -109$ kcal/mol (Scheme 2A).

The ca. 100 kcal/mol difference in the NMe_3^+ substituent effect on oxygen-deprotonation of glycine in the gas phase and water reflects the much larger free energy of solvation of a trimethyl ammonium cation and a carboxylate anion when these groups are placed at separate molecules, ΔG^R_{solv}, than when they are placed in the same molecule to give a formally neutral zwitterion, ΔG^P_{solv} (Scheme 2). This is because access of solvent to the charged groups at $^+Me_3NCH_2CO_2^-$ is restricted by the covalent attachment of these groups, and because stabilization of $^+Me_3NCH_2CO_2^-$ by interaction with solvent is offset by a *weakening* of the "internal" solvation of the zwitterion by interaction between opposing charges in the polar solvent water compared with the gas phase. The separation between the opposing charges at the carbanion zwitterion is smaller than for the charges at



Scheme 2.

$^{+}Me_3NCH_2CO_2^-$ (Scheme 2B). This should have the effect of reducing ΔG^{P}_{solv} (Scheme 2B) by further restricting access of solvent to charge and by increasing the offsetting "internal" solvation of the zwitterion in the gas phase.

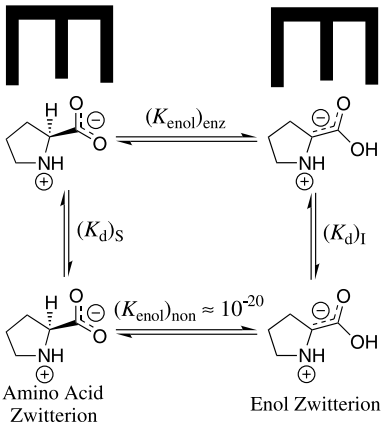
In fact, only a fraction of the large increase in thermodynamic driving force for carbon deprotonation of a cationic amino acid in gas phase compared with solution can be observed for reaction at a nonpolar enzyme active site, because of the following. (1) The "effective" dielectric constant at the enzyme active site will be larger than $D = 1$ for the gas phase so that the *differential stabilization* of the zwitterionic enolate at a nonpolar enzyme active site compared with solution will be smaller than the ca. 100 kcal/mol differential stabilization in the gas-phase compared with water. (2) The uncertain, but probably significant energetic cost of generating the base that deprotonates the amino acid, a thiol anion [21–23], at a nonpolar enzyme active site. (3) The cationic (O-protonated) amino acid is the minor species in solution at pH 7. Therefore, any price *paid* for protonation of the bound substrate at oxygen will need to be subtracted from the increase in the thermodynamic driving force for carbon deprotonation at a nonpolar enzyme active site compared with water.

It is important to emphasize that only a small part of the >100 kcal/mol differential stabilization of the zwitterionic carbanion in the gas phase compared with water need be recovered during enzyme-catalyzed formation of a zwitterionic enolate at a nonpolar active site in order for this to make a significant contribution to the rate acceleration for the enzymatic reaction. For example, a value of just 10% of this maximum could result in a rate increase for deprotonation of the carbon acid of $>10^7$-fold.

2.2. Mechanism for the enzyme-catalyzed racemization of amino acids

The large difference between the catalytic performance of amino acid racemases and that of other enzymes that catalyze the deprotonation of α-carbonyl carbon is not widely appreciated. The deprotonation of dihydroxyacetone phosphate is relatively "easy" and occurs with a halftime of ca. 30 min in the presence of 0.8 M 3-quinuclidinone buffer at pH 7.5 [24], while the estimated halftime for deprotonation of proline zwitterion under the same reaction conditions is ca. 1500 years [17]. Since there is no large difference in the kinetic parameters for the reactions catalyzed by proline racemase and triosephosphate isomerase, there must be a substantially larger rate acceleration of the former enzymatic reaction [17].

We have estimated that proline racemase provides ca. 19 kcal/mol stabilization of the transition state for deprotonation of proline, and proposed that this stabilization is achieved partly or entirely by reducing the large 27 kcal/mol thermodynamic barrier to nonenzymatic conversion of the amino acid zwitterion to the zwitterionic enol ($K_{enol} \approx 10^{-20}$, Scheme 3) [17]. Enolization of the proline zwitterion results in a substantial *decrease* in the separation between the interacting positive and negative charges. The stabilizing intramolecular electrostatic interaction between charges will increase with decreasing dielectric constant, and this will favor the evolution of a nonpolar enzyme active site.



Scheme 3.

The effective dielectric constants (ε_{eff}) for the interior of a protein, calculated from the perturbation by the protein of the acidity constants, determined in water, for deprotonation of amino acid side chains determined in water show considerable variation [2,4]. Refinements in these calculations suggest that protein reorganization associated with formation of charged groups at the protein interior may cause a significant increase in ε_{eff} that is not accounted for in some calculations, but corrections for the effect of protein reorganization still gives values of ε_{eff} for the protein interior that are well below the value for water [1]. The dielectric constants of the interior of several proteins, calculated from molecular dynamic simulations in water is low ($D = 2$–3) for protein interiors from which charged groups have been excluded and larger ($D = 11$–21) for the protein molecule as a whole [3]. There is a tendency for ε_{eff} to increase on moving from the protein interior to the protein surface where the majority of charged amino side chains are found [3]. The results of these calculations are consistent with the notion that the effective dielectric constant at enzyme active sites which lie at the bottom of deep pockets in the protein is lower than active sites at solvent-exposed clefts; and, that the effective dielectric constant of both types of active site is lower than for water.

Glutamate racemase catalyzes the interconversion of D- and L-glutamate. The enzyme is composed of two identical subunits of molecular weight 28,000. X-ray crystallographic analysis of glutamate racemase shows that the substrate analog glutamine is bound at a deep pocket that is formed by contacts between hydrophobic residues of the subunit dimers [25]. Substrate binding at such a deep pocket that is sequestered from the solvent should occur in a medium with a bulk dielectric constant that is substantially smaller than for water. Also present at this active site are a thiol anion and thiol from Cys-70 and Cys-178 which participate in nonstereospecific proton transfer at glutamate [26], along with two carboxylic acids from Asp-7 and Glu-147, an imidazole side chain from His-180 and a hydroxyl from Ser-8 all of which are assumed to participate in stabilization of the transition state for proton transfer [25,27].

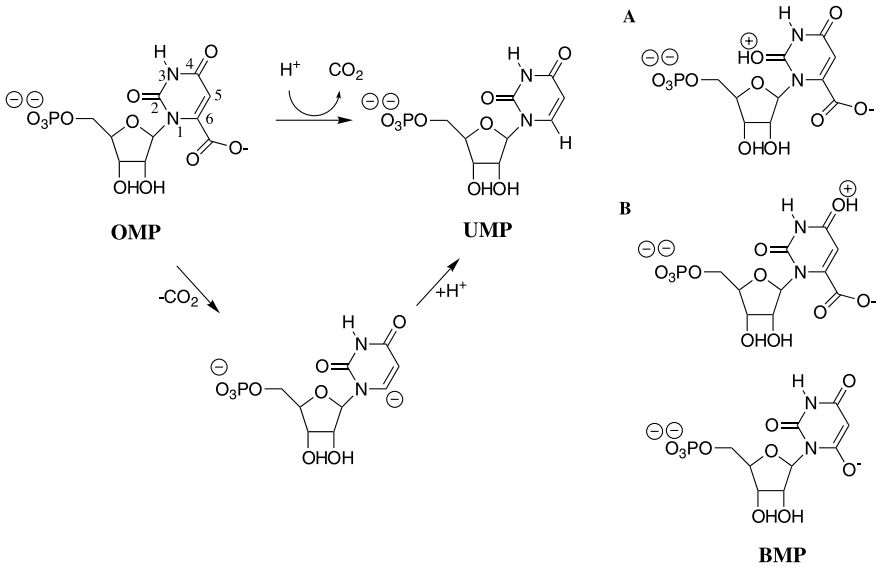
It is curious that enzymes which catalyze the racemization [21,26] or epimerization [23] of amino acids use a pair of thiol side chains of cysteine for nonstereospecific proton transfer at the bound substrate. This is because thiolate anions are poor catalysts of deprotonation of carbon in aqueous solution. For example, the second-order rate constant for deprotonation of a model ketone by $HOCH_2CH_2S^-$ is 30-fold smaller than that for deprotonation by a substituted phenoxide anion of the same pK_a [28]. It is interesting to speculate on how the advantages of thiol/thiolate side chains for catalysis of amino acid-racemization at an active site of low dielectric constant might outweigh the advantages of carboxylic acid/carboxylate side chains, which are the catalytic base of choice in some enzyme-catalyzed proton transfer reaction at carbon [29–31].

A reduction in the dielectric constant of an enzyme active site below that of aqueous solution will cause an increase in the driving force for deprotonation of cationic carbon acids by an anionic base by specifically stabilizing the carbanion zwitterion through enhancement of electrostatic interactions between opposing charges (see above), and by causing an increase in the basicity of the anionic amino acid side chain that accepts the proton from substrate. Carboxylate anions are more strongly stabilized by solvation than are thiolate anions, and the transfer of a carboxylate compared to a thiolate anion to this nonpolar enzyme active site will therefore result in a larger increase in the basicity of the former [32] that will favor deprotonation of carbon acids by carboxylate anions. At the same time, there is the problem of burying anions at the nonpolar enzyme active site which is more serious for the more strongly solvated carboxylate anion. This problem may presumably be solved by placing additional polar groups at the active site to stabilize charge at the carboxylate ion, but this will act to increase in the effective dielectric constant of the enzyme active site. We are uncertain as to whether the problem can be *easily* solved.

In summary, the observation that enzymes which catalyze the racemization and epimerization of amino acids use a thiolate anion rather than a carboxylate anion in catalysis of deprotonation of α-amino carbon buried in a deep active site pocket is consistent with the notion that: (1) a substantial rate acceleration can be obtained simply through the stabilization of the zwitterionic carbanion intermediate by the enhancement of intramolecular electrostatic interactions between closely spaced opposing charges at an active site of low dielectric constant. (2) Enzymatic catalysis of deprotonation of carbon by thiolate anions is favored over deprotonation by carboxylate anions in spite of the lower basicity and intrinsic catalytic activity of the former at a nonpolar enzyme active site, because thiolate anions are is some sense more *compatible* than strongly solvated oxyanions with an active site of low dielectric constant.

3. Orotidine monophosphate decarboxylase

Orotidine monophosphate decarboxylase (OMP decarboxylase, Scheme 4) catalyzes the final step in the de novo biosynthesis of uridine monophosphate (UMP). This enzyme has attracted much attention in recent years. It uses no metal ion or

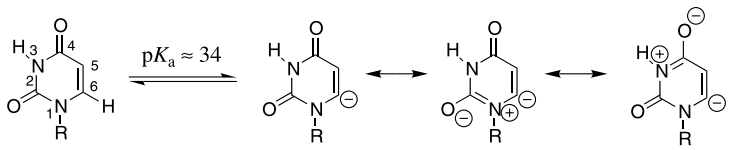


Scheme 4.

cofactors [33–37], yet shows a remarkable proficiency [38] in catalysis of a reaction for which it has been difficult to obtain experimental evidence for chemically plausible mechanisms that utilize amino acid side chains in their conventional roles of Brønsted acid/base or nucleophilic catalysts [39,40].

Direct decarboxylation of UMP gives the vinylic C-6 carbanion of UMP (Scheme 4), which is considered unattractive because of its high instability. It has been proposed that OMP decarboxylase proceeds by a mechanism in which protonation of C-6 by an ammonium ion side chain of lysine is concerted with decarboxylation, so that formation of the unstable intermediate is avoided [35]. However, there is no chemical precedent for such a concerted decarboxylation, or experimental or computational evidence that there is an energetic advantage to coupling proton transfer and decarboxylation in a concerted reaction to counter arguments from simple theoretical considerations that there is a normally a preference for the competing stepwise reaction [41].

Alternatively, it has been proposed that OMP decarboxylase stabilizes the transition state for formation of the C-6 carbanion by protonation at O-2 (**A**, Scheme 4) [42], and that an even larger stabilization might result by protonation at O-4 (**B**, Scheme 4) [43]. Such proton transfer will only provide significant stabilization of the transition state for decarboxylation to form the carbanion reaction intermediate when there is a significant thermodynamic driving force for protonation of the amide-type oxygens at O-2 and O-4 at this intermediate [44]. This would require a substantial increase in the basicity of these oxygen at the enzyme-bound substrate compared with water and this might be the case for a reaction at a nonpolar enzyme active site [43]. However, an X-ray crystal structure of a complex between OMP decarboxylase and the transition state analog 6-hydroxyuridine 5′ phosphate (BMP) shows only the presence of hydrogen bonds from weakly acidic amide nitrogen to

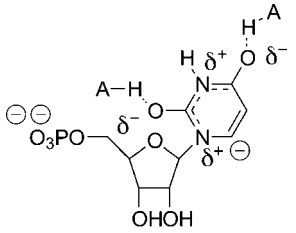


Scheme 5.

O-2 and O-4 [34]. This provides strong evidence that there is no significant stabilization of the transition state for decarboxylation from concerted proton transfer to these carbonyl oxygens.

The putative intermediate of the OMP decarboxylase is a vinylic carbanion. However, the pK_a of 34 that has been estimated for deprotonation of uridine at C-6 [45] is 10 units lower than the pK_a of 44 estimated for deprotonation of a simple alkene [46]. This shows that the substituents at the uridine ring provide considerable stabilization of negative charge at C-6. This is partly or mainly due to intramolecular interactions between negative charge at C-6 and the partly positively charged N-1 and N-3 (Scheme 5). The X-ray crystal structure of a complex between OMP decarboxylase and BMP shows that the amide side chain of Gln215 and the amide nitrogen of Ser 154 are positioned to form hydrogen bonds to O-2 and O-4, respectively, of the transition state analogue. Similar hydrogen bonds to O-2 and O-4 of the putative carbanion reaction intermediate would increase the contribution of zwitterionic valence bond resonance structures, with formal positive charge at N-1 and N-3, to the structure of this intermediate.

X-ray crystallographic analysis of the complex between OMP decarboxylase and the putative transition state analog BMP shows that binding of this ligand is accompanied by protein loop movements that nearly completely envelop the ligand, so that the effective dielectric constant of the enzyme active site should be substantially reduced compared with that of solvent. This reduction in the dielectric constant of the active site favors formation of hydrogen bonds between enzyme and O-2 and O-4, and the development of stabilizing intramolecular electrostatic interactions between opposing negative charge and partial positive charge at the ring nitrogen of the putative zwitterion-like reaction intermediate **I**.



I

The rate acceleration of the OMP decarboxylase-catalyzed reaction is so large that it is unlikely to be accounted for by the simple stabilization of a putative vinyl carbanion reaction intermediate [38]. A combined quantum–mechanical and molecular

mechanical study provides evidence that OMP-decarboxylase uses the intrinsic binding energy of the phosphate group of substrate to hold the reactive carboxylate group close to a highly anionic region of the active site [36]. The result is a reduction in the activation barrier for catalysis because the electrostatic destabilization of the reaction ground state is specifically relieved at the transition state for enzyme-catalyzed decarboxylation, where the charge at the carboxylate fragment is presumably close to zero, and the net difference in energy of the ground and transition state is smaller than in the absence of electrostatic destabilization of reactant.

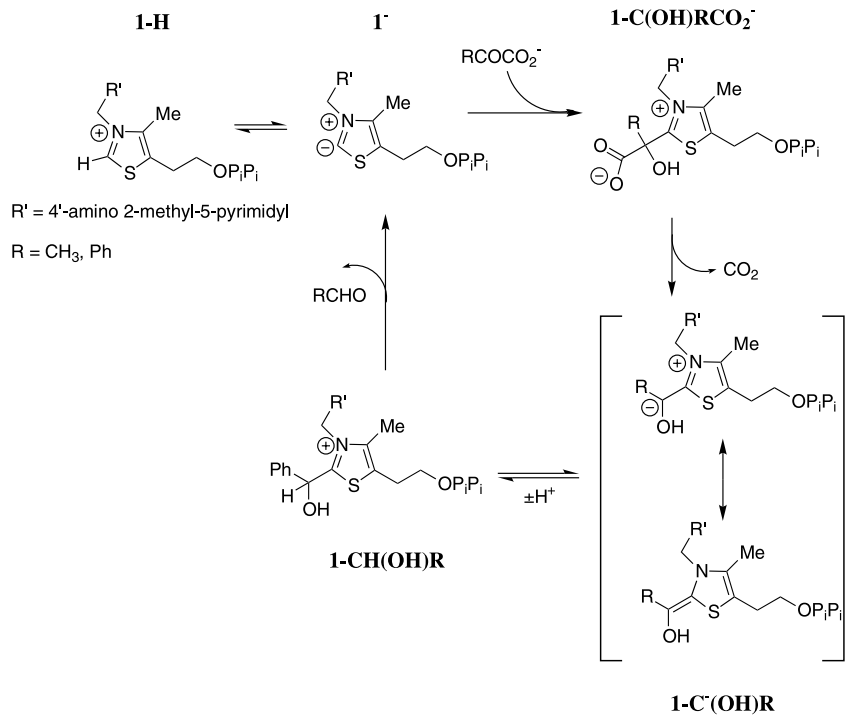
In summary, we propose that conventional Brønsted acid–base catalysis in which there is formal proton transfer between enzyme and substrate makes no significant contribution to the rate acceleration for the OMP decarboxylase-catalyzed reaction; and, that this catalyst instead provides an active site with the following features favorable to facile decarboxylation of substrate.

(1) A nonpolar environment that allows the development of strong electrostatic interactions that destabilize the reactive carboxylate anion of bound OMP and stabilize zwitterion-like vinylic carbanion reaction intermediate.
(2) The placement of cationic amino acid side chains to show optimal stabilizing interactions with the substrate phosphate group when the reactive carboxylate anion is moved closed to anionic amino acid side chains [36]. In other words, the intrinsic binding energy of the substrate phosphate group is *utilized* to drive the development of electrostatic stress in the ground state that is relieved in the transition state for decarboxylation [47].
(3) The placement of hydrogen bond donors at O-2 and O-4 of OMP. This provides specific stabilization of the transition state for decarboxylation through the strengthening of hydrogen bonds that occurs with the development of negative charge at C-6 at the transition state, and through the (proposed) tendency of these hydrogen bonds to enhance stabilizing intramolecular electrostatic interactions at the zwitterion-like intermediate.

4. Pyruvate decarboxylase

Pyruvate decarboxylase catalyzes the decarboxylation of pyruvate to form acetaldehyde. There is a good evidence that the solution [15,16] and enzyme-catalyzed reactions [48] follow similar pathways (Scheme 6, $R = CH_3$) where the ylide $\mathbf{1}^-$ formed by deprotonation of thiamine pyrophosphate (**1-H**) adds to the keto group of pyruvate to form **1-C(OH)RCO$_2^-$**. This adduct undergoes decarboxylation to form a second ylide (**1-C$^-$(OH)R**) which is next protonated and then cleaved with loss of acetaldehyde to regenerate $\mathbf{1}^-$. Pyruvate decarboxylase requires a Mg^{2+} cofactor, which has been shown by X-ray crystallographic analysis to coordinate to the enzyme and pyrophosphate group of bound substrate [49,50].

X-ray crystallographic determination of an enzyme crystal structure may place everything needed to explain catalysis by the examined enzyme in plain view. At the same time, these structures raise questions about whether one is gifted enough to *see* in cases such as pyruvate decarboxylase where there are a scarcity of amino acid side

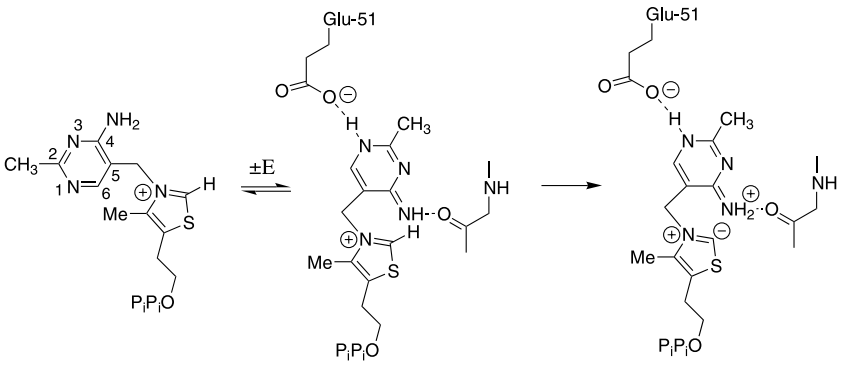


Scheme 6.

chains to participate in Brønsted acid–base catalysis. There are two revealing observations from the X-ray crystal structure of this enzyme that are relevant to the origin of the rate acceleration for the catalyzed reaction.

(1) "The catalytic centers containing both thiamine diphosphate and Mg(II) are located deep in the intermononer interface within each dimer" [49]. The protein dielectric constant will decrease with decreasing distance from the protein surface. Therefore, much or all of the rate acceleration for pyruvate decarboxylase may be associated with the low effective dielectric constant for the enzyme active site as was first suggested 35 years ago [14–16]. This low dielectric constant will act to stabilize the two zwitterionic intermediates $\mathbf{1}^-$ and $\mathbf{1\text{-}C(OH)RCO_2^-}$ relative to **1-H**, as discussed above for amino acid enolate zwitterions. In addition, catalysis might result because of ground state destabilization associated with placement of the carboxylate anion of $\mathbf{1\text{-}C(OH)RCO_2^-}$ at the nonpolar active site that is relieved upon decarboxylation to form $\mathbf{1\text{-}C^-(OH)R}$.

(2) The first step in the decarboxylation reaction catalyzed by pyruvate decarboxylase is deprotonation of the thiazolium ring of **1-H**, but the protein catalyst provides no basic amino acid side chain to accept this proton. Therefore, the observation that the thiamine cofactor is bound in a conformation where the projection of the thiazolium and aminopyrimidine rings from the bridging methylene group defines a "V" (Scheme 7) is particularly significant [49–53].



Scheme 7.

This V conformation is "virtually never" [50] observed in the many relevant crystal structures of thiamine analogues. On the other hand, it is the conformation of choice for thiamine pyrophosphate bound at enzyme active sites which like, pyruvate decarboxylase, lack a basic amino acid side chain to abstract a proton from the thiazolium ring. This conformation brings the basic 4′-amino nitrogen of the pyrimidine ring into a position where it may act as a Brønsted base to abstract a proton from the thiazolium ring (Scheme 7). It has been proposed that this cofactor is bound as the imino tautomer, where the proton at N1′ is stabilized by hydrogen bonding with the carboxylate anion of Glu-51, and the 4′-imino cation is stabilized by hydrogen bonding to the carbonyl oxygen of Gly-413 [49,50]. Intramolecular deprotonation of **1-H** (Scheme 7) is attractive compared with deprotonation by an amino acid side chain for a cofactor buried at a nonpolar active site, because this eliminates problems associated with burying a charged amino side chain at this nonpolar milieu.

The reduced affinity of E91D mutant pyruvate decarboxylase for thiamine pyrophosphate allows the preparation of the mutant apoenzyme, and the study of its reactions with exogenous cofactor. The following results of experiments on this mutant enzyme establish an important link between the low dielectric constant for the active site of pyruvate decarboxylase and the perturbation of the equilibrium constant for deprotonation of the benzaldehyde adduct to thiamine pyrophosphate **(1-CH(OH)R)** [**R** = Ph, Scheme 6] to form the carbanion zwitterion (**1-C$^-$(OH)R**) [48].

(1) The maximum wavelength for fluorescent emission of the thiamine pyrophosphate analog thiochrome diphosphate bound to pyruvate decarboxylase falls between 422 and 424 nm for excitation at 290 nm. There is a good linear correlation between the emission maximum for thiochrome diphosphate in 1-alkanol solvents and the solvent dielectric constant. The maximum of 422–424 nm observed for fluorescence of the enzyme-bound cofactor analog falls between the maximum for fluorescence in 1-pentanol and 1-hexanol, so that the *apparent* dielectric constant of the enzyme active site lies between the values of $D = 13$–15 for these solvents.

(2) Mixing of the E91D mutant apoenzyme with **1-CH(OH)R [R** = Ph, Scheme 6] at pH 6.1, is accompanied by an increase in absorbance at 380 nm due to the

relatively slow deprotonation of the enzyme-bound **1-CH(OH)R** to form the carbanion zwitterion **1-C$^-$(OH)R**. A pK_a of 15.4 has been determined for deprotonation of **1-CH(OH)R** in water [54,55]. Therefore, the accumulation of the carbanion zwitterion **1-C$^-$(OH)R** at the active site of pyruvate decarboxylase at pH 6.1, provides direct evidence that the binding of this adduct to pyruvate decarboxylase is accompanied by a >9 units increase in the pK_a for carbon deprotonation. This correlation between the low apparent dielectric constant of pyruvate decarboxylase and the perturbation in the pK_a of bound **1-CH(OH)R** is simple and compelling. However, it is important to emphasize that enzymes provide active sites with complex microenvironments that favor their catalyzed reactions, so that in our opinion it would be hasty to conclude the entire perturbation in the pK_a of **1-CH(OH)R** bound to pyruvate decarboxylase can be attributed to a single bulk property of the enzyme active site.

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