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Invited minireview

Interplay of organic and biological chemistry in understanding coenzyme mechanisms: example of thiamin diphosphate-dependent decarboxylations of 2-oxo acids

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Abstract With the publication of the three-dimensional structures of several thiamin diphosphate-dependent enzymes, the chemical mechanism of their non-oxidative and oxidative decarboxylation reactions is better understood. Chemical models for these reactions serve a useful purpose to help evaluate the additional catalytic rate acceleration provided by the protein component. The ability to generate, and spectroscopically observe, the two key zwitterionic intermediates invoked in such reactions allowed progress to be made in elucidating the rates and mechanisms of the elementary steps leading to and from these intermediates. The need remains to develop chemical models, which accurately reflect the enzyme-bound conformation of this coenzyme.

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Key words: Thiamin diphosphate; 2-Oxo acid decarboxylase; Enzyme model; Zwitterionic intermediate

1. Introduction

The advent of X-ray crystallographic structure determinations of proteins, in conjunction with the ability to make substitutions at specific sites of the protein employing the powerful capabilities of modern molecular biology, provides an excellent opportunity to evaluate the additional catalytic effects of the protein on coenzyme-dependent enzymatic reactions. The reactions of many coenzymes derived from water-soluble vitamins have been mimicked in model systems and one can now evaluate whether or not these chemical models are true representations of the process carried out by the coenzyme when protein-bound. Such comparisons of enzymatic and model chemical systems for the same reaction also enable us to test current theories of enzyme catalysis.

In this review recent developments are summarized in our understanding of how one of the best-known water-soluble vitamins (coenzymes), thiamin (the coenzyme is thiamin diphosphate, ThDP), carries out its functions. We are attempting to identify whether and by how much the protein accelerates specific steps in such reactions. The reactions of ThDP include: non-oxidative reactions (reactions B, G, Scheme 1), such as those of pyruvate decarboxylase (PDC) and benzoylformate decarboxylase (BFD) [1,2] producing acetaldehyde and benzaldehyde; and oxidative pathways (reactions C–F, Scheme 1), such as the pyruvate oxidases (POX) using flavin as the oxidant to produce acetate [3] or acetyl phosphate [4], the pyruvate dehydrogenase multienzyme complex (PDHc) and its family of enzymes which utilize lipoic acid as the oxidant to produce acetyl-CoA [5]; and pyruvate-ferredoxin oxidoreductases (PFOR) which use Fe_4S_4 cluster chemistry to produce acetyl-CoA [6].

2. On the mechanisms of proton transfers

As exemplified with PDC and BFD (Scheme 2), there are several steps where proton transfers are called for. Crystallographic evidence [2,4,6–9] strongly suggests that the 4′-amino group participates in abstraction of the proton from the C2 position (reaction A, Scheme 1), perhaps involving a rare imino tautomer stabilized by three highly conserved hydrogen bonds to N1′, N3′ and N4′H_3, (denoting the proton bonded to N4′ on the N3′ side of the pyrimidine ring). The participation of the aminopyrimidine in this manner had been suggested more than 20 years ago [10]. Are the relevant pK_as balanced for rapid proton transfer? The pK_as for the conserved Glu (across from the N1′ atom) and at N1′ are likely to be similar, while the pK_a for ionization of the amino group at N4′H_5 (denoting the proton bonded to N4′ on the C5′ side of the pyrimidine ring) once the N1′ atom is protonated is ca. 12 [10], and for ionization of C2H is 17–19 [11]. The special environment of the V coenzyme conformation present in all of these enzymes [12] assures that for a reasonable distance between N4′ and C2, proton transfer would take place at a rate exceeding the turnover number for many such enzymes, i.e. 60–100 s^{-1}. This is important in view of the C-13 NMR report on PDC, claiming that C2H of the bound ThDP is undissociated at pH 6.0 [13]. Only recently a method was developed to generate the conjugate base ylide/carbene in a reproducible fashion [14], and preliminary results have already shown that it is very reactive with benzaldehyde [15], confirming Breslow's proposals for thiamin action [16]. Model studies also showed that the ylide/carbene reacts with benzaldehyde via a nucleophilic addition, and no evidence of reactions typical of carbenes, such as insertion, could be found [17,18].

To examine the rates of the steps leading to and from the C2α-carbanion/enamine in Scheme 1, in this laboratory the enamine is being generated by the addition of base to a C2α-hydroxybenzylthiazolium or C2α-hydroxyethylthiazolium salt [19,20], then measuring either the proton transfer or oxidation rates. First, the pK_a at C2α (reaction B, Scheme 1) was measured in pure DMSO [21]. By direct observation of the enamine generated in a stopped-flow spectrophotometer,

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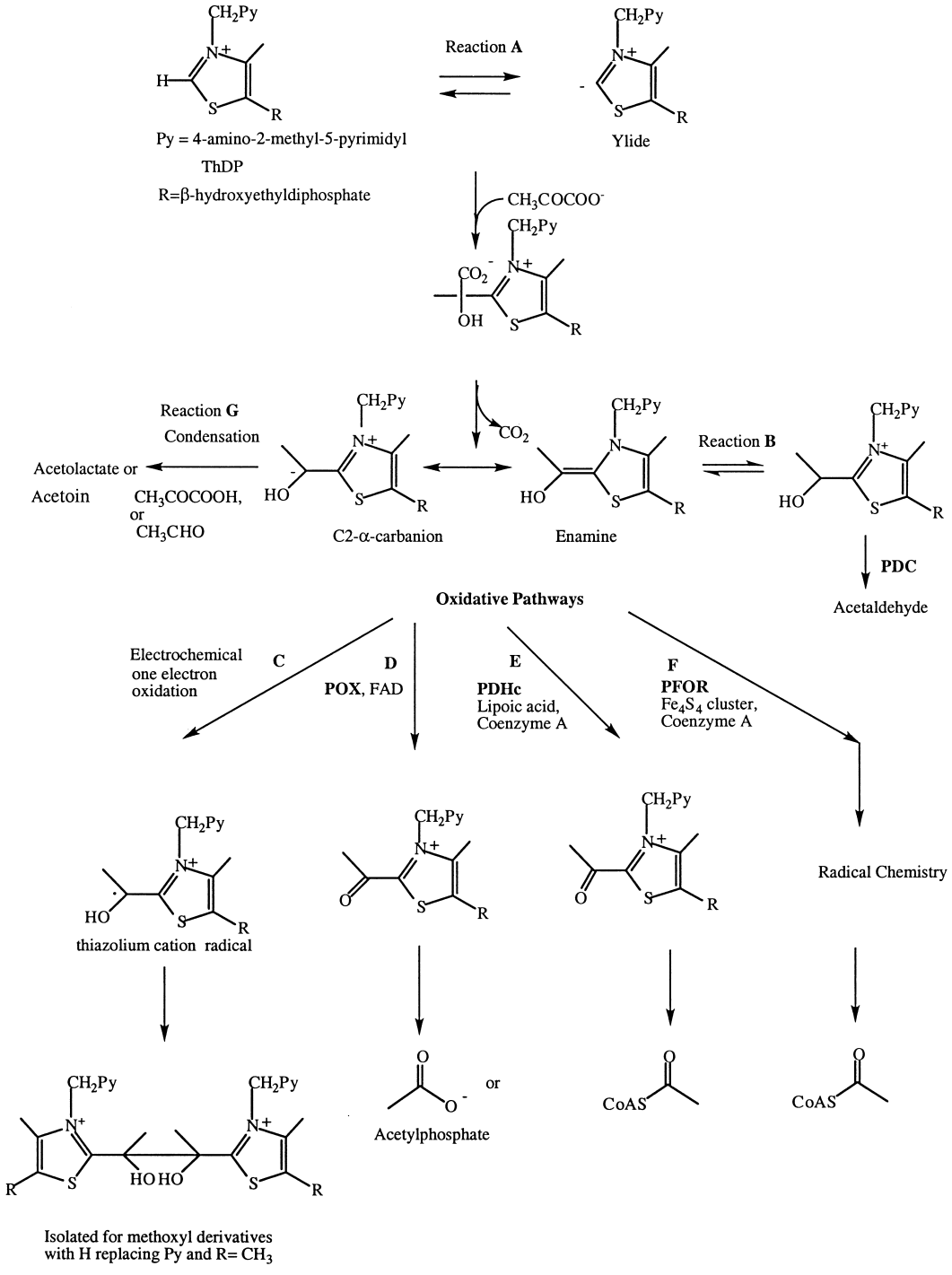
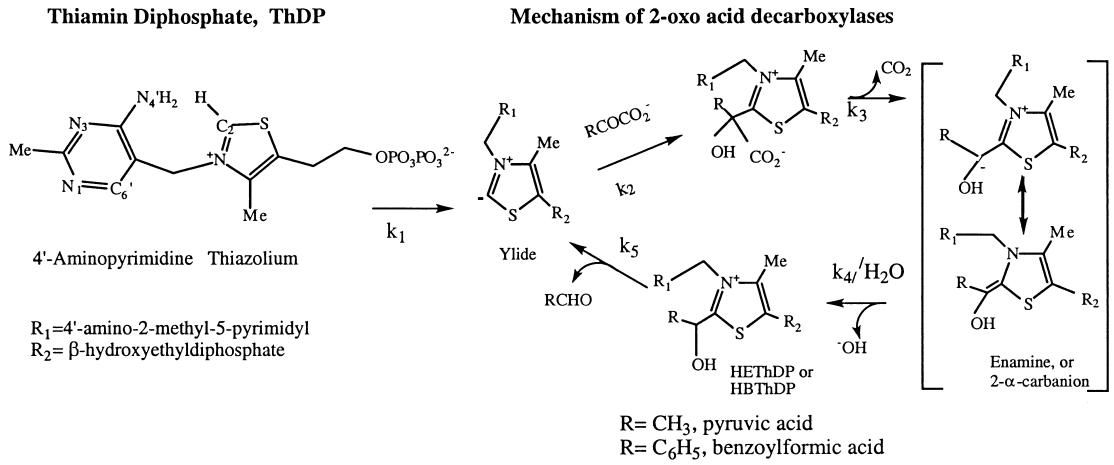


Scheme 1.

the rate constants for reversible proton dissociation at the C2α position could also be measured in water. The pK_a is between 15 and 16 [22,23] for C2α-hydroxybenzylthiazolium salt, and is 15–16 in 32–37 mol% DMSO for C2α-hydroxyethylthiazolium salt (extrapolates to approximately 18 in water) [24]. There is a substantial primary deuterium kinetic isotope effect for the deprotonation reaction [4–6]; the rate constant for reprotonation of the enamine/C2α carbanion by water is many orders of magnitude below diffusion control. It was concluded that PDC and BFD assist in the protonation of the enamine to afford rate constants commensurate with enzymatic turnover numbers [23,24].

How do ThDP enzymes solve this high pK_a problem? The following experiment provides some of the answers to this riddle. When the E91D variant of yeast apo-PDC (EC 4.1.1.1) was exposed to C2α-hydroxybenzyl-ThDP (HBThDP, Scheme 2), this putative intermediate was partitioned on the enzyme between release of the benzaldehyde product (evidenced by regeneration of active enzyme), and dissociation of the proton at C2α to form the enamine/C2α-carbanion



Scheme 2.

intermediate (evidenced by the appearance of the visible spectrum of the intermediate). While the pK_a for this dissociation is ~15.4 in water, formation of the enamine at pH 6.0 on the enzyme indicates a greater than nine units pK_a suppression by the enzyme environment [25]. The fluorescence emission properties of thiochrome diphosphate, a fluorescent ThDP analog and a competitive inhibitor for PDC, when PDC-bound, resembles that observed in 1-pentanol and 1-hexanol, suggesting an apparent dielectric constant of 13–15 for the PDC active center. Such a low effective dielectric constant could account for much of the observed >9 units pK_a suppression at the C2α position for ionization of HBThDP. The dramatic stabilization of this (and presumably other) zwitterionic intermediate(s) is sufficient to account for as much as a 10^9-fold rate acceleration on PDC, providing the bulk of the rate acceleration by the protein over and above that afforded by the coenzyme.

3. Consequences of oxidation of the enamine

The enamine was first generated under electrochemical conditions. According to both electrochemical results and isolation of the products, there is a one-electron oxidation via a radical cation intermediate (reaction C, Scheme 1) [26]. More recently, electrochemical experiments starting with thiazolium compounds and aldehydes enabled the group of Fukuzumi to observe this radical cation directly by ESR [27].

4. Models for flavin-catalyzed oxidation of the central enamine (reaction D, Scheme 1)

The enzyme POX has dual functions: it produces acetyl phosphate in *Lactobacillus plantarum* [4] and acetic acid in *Escherichia coli* [3]. Models for oxidation at the C2α position by flavin have yielded the following information: (1) there is a need for both a base, and for a free hydroxyl group at the C2α position; (2) surprisingly, the model reaction can proceed by both a one-electron [28] and two-electron pathway since a 5-deazaflavin analogue also acts as an oxidizing agent (presumably proceeding by hydride transfer, more analogous to NAD-dependent oxidations), albeit much more sluggish than flavin [24]; (3) the second-order rate constant for oxidation of the enamine with flavin is $>6000\ s^{-1}\ M^{-1}$, requiring a relatively modest 'effective concentration' of the enzyme [28]. The principal function of the protein is to bring FAD and ThDP into close proximity on POX. The crystal structure of POX [4] indicates that the arrangement of the cofactors is inappropriate for hydride transfer, hence the models suggest a one-electron transfer for the enzymatic reaction.

5. Models for lipoic acid-catalyzed oxidation of the central enamine (reaction E, Scheme 1)

In the family of 2-oxo acid dehydrogenase multienzyme complexes, there are three proteins (named E1, E2 and E3) charged with the overall reaction generating acyl-coenzyme A and NADH as products. It is generally accepted that lipoyl-E2 (lipoic acid is covalently amidated onto a Lys of E2) is the oxidizing agent for the enamine product of decarboxylation non-covalently bound to E1. Incisive experiments from Frey's labs [29] suggested that the redox process and acyl transfer are distinct steps in the mechanism. Earlier models could only demonstrate reaction between the enamine and linear disulfides, but not with lipoic acid [30]. Our first model based on pre-generation of the enamine indicated that: (1) the enamine was virtually unreactive with lipoic acid in aprotic media; (2) addition of Hg compounds (presumably to trap the sulfide) would lead to a modest reactivity; (3) a C2α-methoxyl derivative was reactive under these circumstances [31]. It was concluded that the protein must play an important function, probably by the addition of an electrophile, such as a proton via a general acid, to one of the sulfur atoms of the lipoic acid, thereby shifting the equilibrium to the products. Next, methyl *S*-methyllipoate (forming thiosulfonium salts) was synthesized to mimic the positive charge created by protonation of lipoic acid. The diastereomeric pair of methyl *S*-methyllipoates (from D,L-lipoic acid methyl esters) was then added to the enamine generated from C2α-methoxybenzylthiazolium salts giving the following information [32]: (1) there is a tetrahedral intermediate formed between the *S*-methyllipoate and the enamine (evidenced by NMR and mass spectrometry); and (2) most importantly, the second order rate constant for the reaction is $6.6\times10^4\ s^{-1}\ M^{-1}$, perhaps 10^8 times faster than with unactivated lipoic acid. The results led us to speculate that when the enamine-E1 complex reacts with lipoamide-E2, there is a tetrahedral intermediate formed, for an

instant cross-linking E1 and E2, with the assistance of a general acid catalyst located on E1 near the ThDP site. An important conclusion from these chemical models is that the protein surrounding it must activate lipoic acid, while FAD is reactive enough to need no additional activation by the protein.

6. Preliminary model studies for the enzyme acetoin dehydrogenase

Acetoin dehydrogenase is a multienzyme complex, very similar to the 2-oxo acid dehydrogenase multienzyme complexes [33]. It consists of three proteins, E1 (again, it has ThDP), while E2 and E3 are analogous to those found in E2 and E3 in the 2-oxo acid dehydrogenases, i.e. with lipoic acid and FAD, respectively. The reaction couples the reverse of reaction G to reaction E in Scheme 1. To study this fascinating retro-benzoin type reaction catalyzed by acetoin dehydrogenases, both an intramolecular bis-coenzyme, as well as the corresponding intermolecular system were tested [15]. Because of the much higher reactivity of FAD analogues than unactivated lipoic acid in model systems, our first model for this enzyme consists of a thiazolium compound and a 10-methylisoalloxazine ring. In the presence of three required components, a base, a thiazolium salt and isoalloxazine in methanol solvent, benzoin undergoes oxidative retro-benzoin condensation (as evidenced by the bleaching of the isoalloxazine absorption in the Vis spectrum) yielding benzaldehyde and methyl benzoate. Acetoin is much less reactive than benzoin. As a control, we also determined the rate of FAD reduction by benzaldehyde (leads to methyl benzoate as product) to be ca. 70 times slower than the reaction with benzoin.

The faster rate observed for benzoin compared with acetoin suggests that a step leading to the expulsion of benzaldehyde from the thiazolium-benzoin covalent adduct and leading to a highly conjugated enamine is rate-limiting overall, while the redox step is quite fast for either substrate. It is unlikely that addition of benzoin to the thiazolium ylide would be faster than addition of acetoin.

7. Prospects for future work

Perhaps most fundamental to future model studies of ThDP-mediated enzymes is the recognition that the aminopyrimidine ring of thiamin is an integral participant in the enzymatic reactions. A brief overview of the literature of thiamin models reveals that, so far, virtually no model studies have been carried out that properly reflect the active site conformation of ThDP. Given that this enzyme-bound conformation may affect the very first step in all ThDP reactions, one can safely conclude that modeling of ThDP enzymes remains a very fertile field for bioorganic chemists.

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