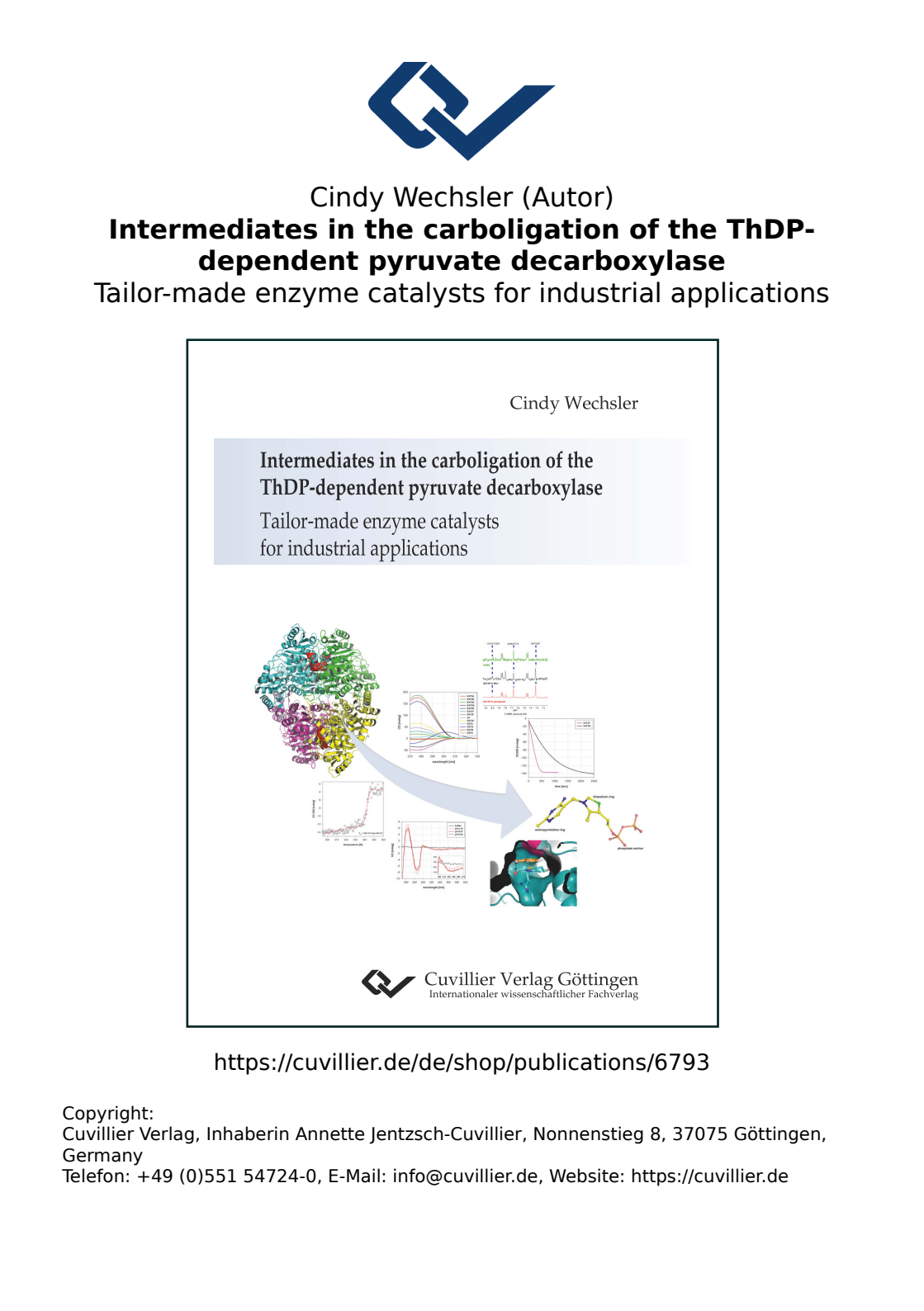

Cindy Wechsler (Autor)

Intermediates in the carboligation of the ThDP-dependent pyruvate decarboxylase

Tailor-made enzyme catalysts for industrial applications

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1 Introduction

1.1 Thiamine and it´s Biological Relevance

Thiamine is a member of the *vitamin B* family. It is commonly known as *vitamin* B_1. Thiamine and its derivatives are a ubiquitous metabolites in all prokaryotes and eukaryotes. In diverse biological pathways they serve as cofactors of essential catalysts such as pyruvate dehydrogenase and transketolase. (Fersht [33], G.F. Combs [37])

Probably due to this metabolic relevance thiamine is intrinsically tied to the discovery and definition of the vitamin's substance class. Back from ancient Chinese literature (about 2600 B.C.) up to the late 19^{th} century a disease called beriberi was repeatedly described in many Asian populations (G.F. Combs [37]). Beriberi is characterized by symptoms of weakened limbs (loss of feeling and strength), irregular heart-rate, edema, emotional disturbance and polyneuritis. In the final stage it might cause cardiac failure and death. Like scurvy and rickets the symptoms were soon known to be linked to a restricted diet. In Asia the main staple food was and still is polished rice. In 1860 first "clinical trials" using different diets amongst sailors of the Japanese navy the prevalence of beriberi could be decreased significantly. In 1897 within a dietary trial in chickens with polished and unpolished rice bran the symptoms of beriberi could be prevented and even cured. Still it was not before 1912 when the first extraction of the so-called "antiberiberi factor" was published and the theory of *"vitamines" - an amine essential for life* - was established (Funk [35]). Even then it took another fourteen years until in 1936 when the chemical formula was published and the name "Thiamine" was scientifically accepted. In 1937 the first commercial production was accomplished. As all living organisms use thiamine in their metabolism but only bacteria, fungi and plants are able to synthesize it, vertebrates depend on the intake of thiamine and other essential vitamins through their diet. Therefore, with the industrial production of vitamins started the widespread fortification of foods with essential nutritional compounds, which has become standard in the developed countries (G.F. Combs [37], Carpenter [20], Kraemer et al. [65], Eggersdorfer et al. [28]).

Today *Vitamin* B_1 deficiency is still common in developing countries, where refined grain and rice remains a central staple food. In developed countries it occurs in alcoholics as well as in children that solely eat snack foods.

Thiamine consists of conjoined pyrimidine and thiazole rings (see fig. 1.1). Free thiamine is easily oxidized to thiamine disulfide and other derivatives. The thiazole hydroxyethyl group can be phosphorylated *in vivo* to form thiamine mono-, di- and triphosphates (G.F. Combs [37]).

Biochemically the most important derivative of thiamine is the thiamine diphosphate (ThDP), also called thiamine pyrophosphate, which is the metabolically active form. ThDP-dependent enzymes are multifunctional biocatalysts and catalyze a broad range of reactions like carboligations, oxidative and non-oxidative decarboxylations, oxidoreductions and the transfer of C_2-fragments (two-carbon units). They are involved in the enzymatic making and breaking of bonds like C–C, C–S, C–N and C–P bonds, but by far the C–C bond formation and cleavage is the most important and prominent example (Tittmann [110], Brovetto et al. [15]). Although ThDP-dependent enzymes catalyze such a wide variety of reactions, the ThDP-mediated catalysis always follows a common path that proceeds via similar cofactor-derived covalent intermediates (Schellenberger [96], Kluger [59], Jordan [50], Jordan and Nemeria [51]).

1.2 Reactivity of ThDP

Albeit the discovery of the structure in the 1930s (Williams [122]) and the intense research on the reaction mechanism, it was not earlier than 1957 when *Breslow* identified the reactive center of the cofactor (Breslow [12], Breslow [13]). As depicted in fig. 1.1 the coenzyme ThDP is composed of two aromatic rings, an 4-aminopyrimidine ring and a thiazolium ring with a diphosphate anchor. This diphosphate anchor facilitates binding of the cofactor to the individual ThDP-dependent enzyme (Hawkins et al. [44], Muller et al. [80]). *Breslow* could show in an deuterium exchange experiment, with the help of an NMR spectrometer, that the formation of the C2-carbanion is the first step in catalysis and crucial to initiate the catalytic cycle. This carbanion or ylide is the actual active form of the cofactor. As carbanion it can perform a nucleophilic attack, e.g. at the α-carbonyl group of pyruvate, resulting in an covalent intermediate.

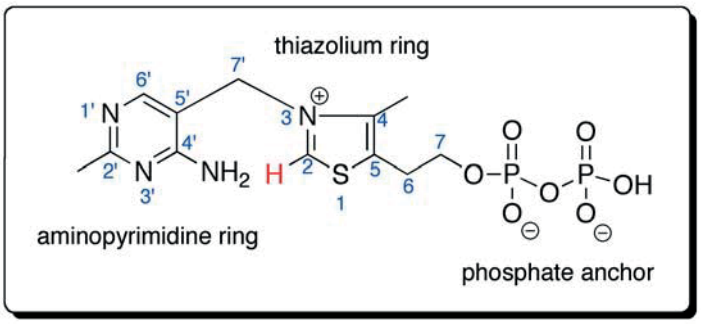

Figure 1.1: **Structure and nomenclature of thiamine diphosphate.** ThDP is composed of a six-membered aminopyrimidine ring and a five-membered thiazolium ring with a diphosphate anchor. The acidic C2-H is shown in red.

Intriguingly, ThDP on its own exhibits only poor catalytic activity under physiological conditions. However, when bound to an ThDP-dependent enzyme, it becomes a powerful catalyst. The low catalytic activity of sole ThDP is a result of its low C2-acidity under physiological pH (pK_A = 17-19, Washabaugh and Jencks [117], Washabaugh and Jencks [118]). Consequentially,

only a small percentage of ThDP exists in the reactive nucleophilic form under these conditions. After binding to an enzyme the proton at the C2-position of the thiazolium ring becomes more acidic, resulting in dramatically increased the rate of C2-deprotonation. (Kern et al. [57], Hübner et al. [47], Jordan et al. [52]).

High efficiency is achieved by ThDP when all chemical moieties comprising the cofactor perform conjointly as catalyst. The ability of the C2-H to ionize and function as a nucleophile or a leaving group with respect to carbonyl addition is the key function (Kluger and Tittmann [60]). The positive charge of the nitrogen of the thiazolium ring promotes this ionization of the C2-carbon by electrostatic stabilization, contributing as electron sink and reducing the negative charge at C2. The ionized carbon is a potent nucleophile that can covalently add to the carbonyl group of a substrate. ThDP covalently binds to the substrate and is able to stabilize a negative charge. The nitrogen atom of the thiazolium ring can also stabilize a negative charge by delocalization on the adducts formed by ThDP with different compounds (e.g. 2-hydroxyethyl-ThDP (HEThDP), see fig. 1.2). The pyrimidine ring contributes as a proton relay to an adjacent glutamate and therefore enables ylide formation, which is crucial for catalysis. The thiazolium functionality allows for the delocalization of the negative charge, hence, the stabilization of the C2α carbanion. The cofactor itself allows for a precise localization of substrate molecules by formation of covalent bonds to its active center (C2). Through the interplay of the cofactor with the enzyme catalysis becomes facilitated while the enzyme provides a binding site for the substrate. This interaction of enzyme and cofactor allows to overcome the entropy problem in a reaction of otherwise two free species (the cofactor and the substrate separately) (Kluger and Tittmann [60], Fersht [33]).

A common motive in ThDP-enzymes is the binding of pyruvate, a central intermediate in aerob and anaerobic metabolism. Pyruvate, or pyruvic acid, supplies energy to living cells via the citric acid cycle when oxygen is present (aerobic respiration) and alternatively reacts to lactic acid by fermentation when oxygen is lacking (Voet et al. [115]). The detailed mechanism of ThDP-mediated pyruvate binding, common to all ThDP-dependent enzymes, is illustrated in fig.1.2. Depending on the enzyme, the carbanion intermediate can undergo different fates. In case of protonation of the HEThDP carbanion, the product acetaldehyde is released, which is the native product formed by pyruvate decarboxylase (PDC). In case of a oxidation of the carbanion, different products can be formed e.g. acetate (pyruvate oxidase (POX)), acetylphosphate (POX/pyruvate-ferredoxin oxidoreductase (PFOR)) or acetyl-dihydrolipoic acid (pyruvate dehydrogenase (PDH)). In case of acetolactate synthase (AHAS) and also in PDC, the carbanion can perform a carboligation and form a new C–C bond, which in PDC is a minor side reaction in competition to the C2α-protonation (Frank et al. [34]).

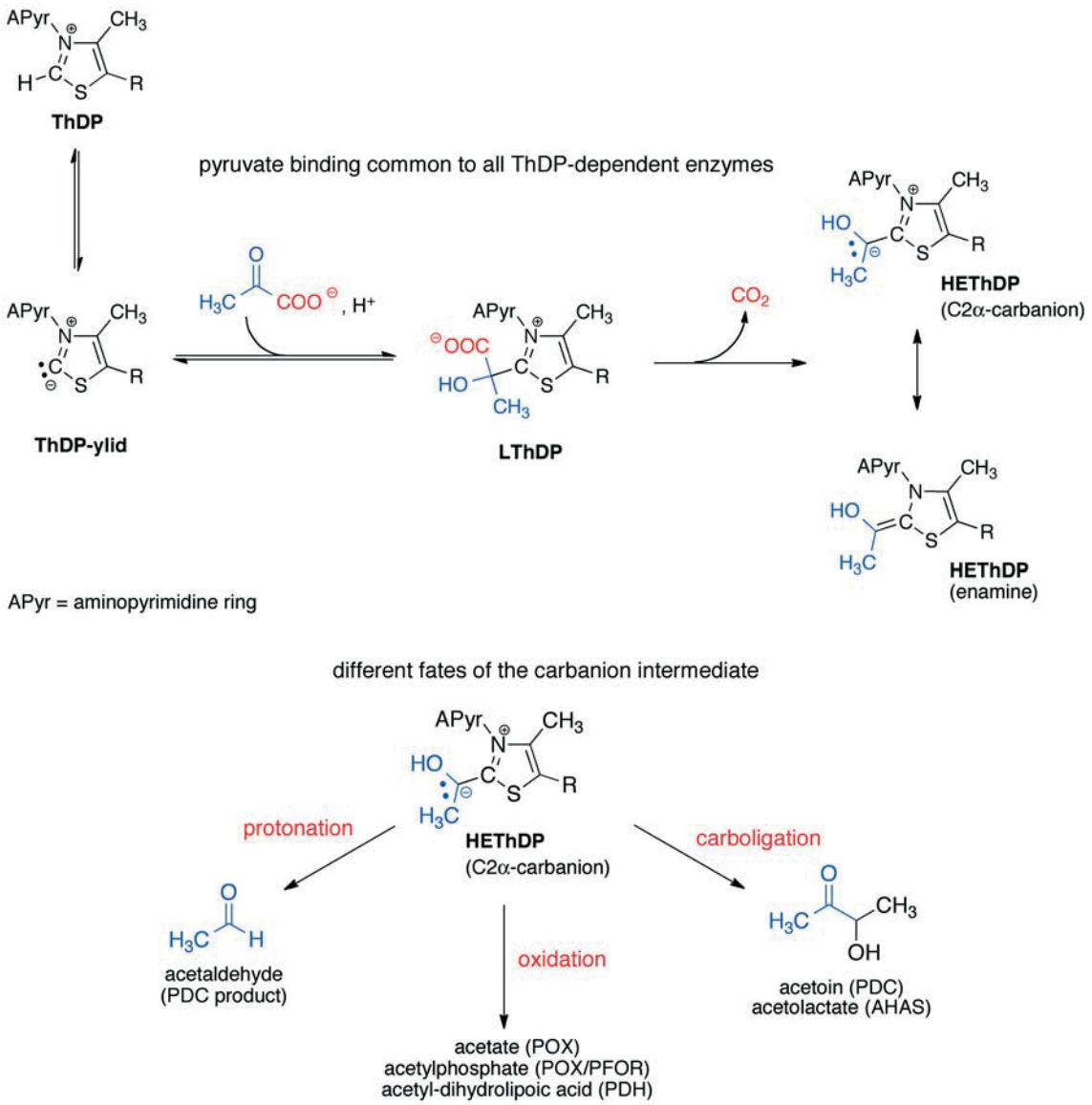

Figure 1.2: **ThDP-mediated pyruvate binding common to all ThDP-dependent enzymes and different fates of the C2α-carbanion.**

As to its chemical properties and innate reactivity as a stable and very potent nucleophile, ThDP in its active carbanion form can be considered nature's very own *N*-Heterocyclic Carbene (NHC) (Berkessel et al. [10]). The so called *'persistent carbenes'* are carbenes that possess an unusually high stability. They are very reactive molecules and are frequently used in numerous synthetic reactions as ancillary ligands in organometallic chemistry, e.g. in transition metal catalysis (for background on NHCs and their chemistry see Herrmann and Koecher [45] and for more detailed applications see Cazin [21]). Indeed thiamine was the first example of a stable carbene, proposed already by *Breslow* in 1957 (Breslow [12]) and for the first time directly observed by structural characterization in a biological system (POX) by *Meyer & Tittmann* (Meyer et al. [77]). In todays research, chemists evoke potential from mimicking ThDP catalysis. They

aim to mimic the active site in enzymes including 'natural NHC' ThDP in order to discover new potent catalysts for asymmetric synthesis. By transferring principal concepts to carbene-catalyzed reactions novel reactions shall be explored. Introducing stereoselectivity and transferring observations from the biological system to 'synthetic' NHC-catalyzed transformations is the ultimate goal (Enders et al. [31], Enders and Narine [30]).

1.3 Activation of ThDP and its Intermediates

ThDP activation is achieved through a distinct tautomeric interplay of the cofactor's chemical moieties with each other and a conserved glutamate in the cofactors periphery. The proposed mechanism is depicted in fig.1.3:

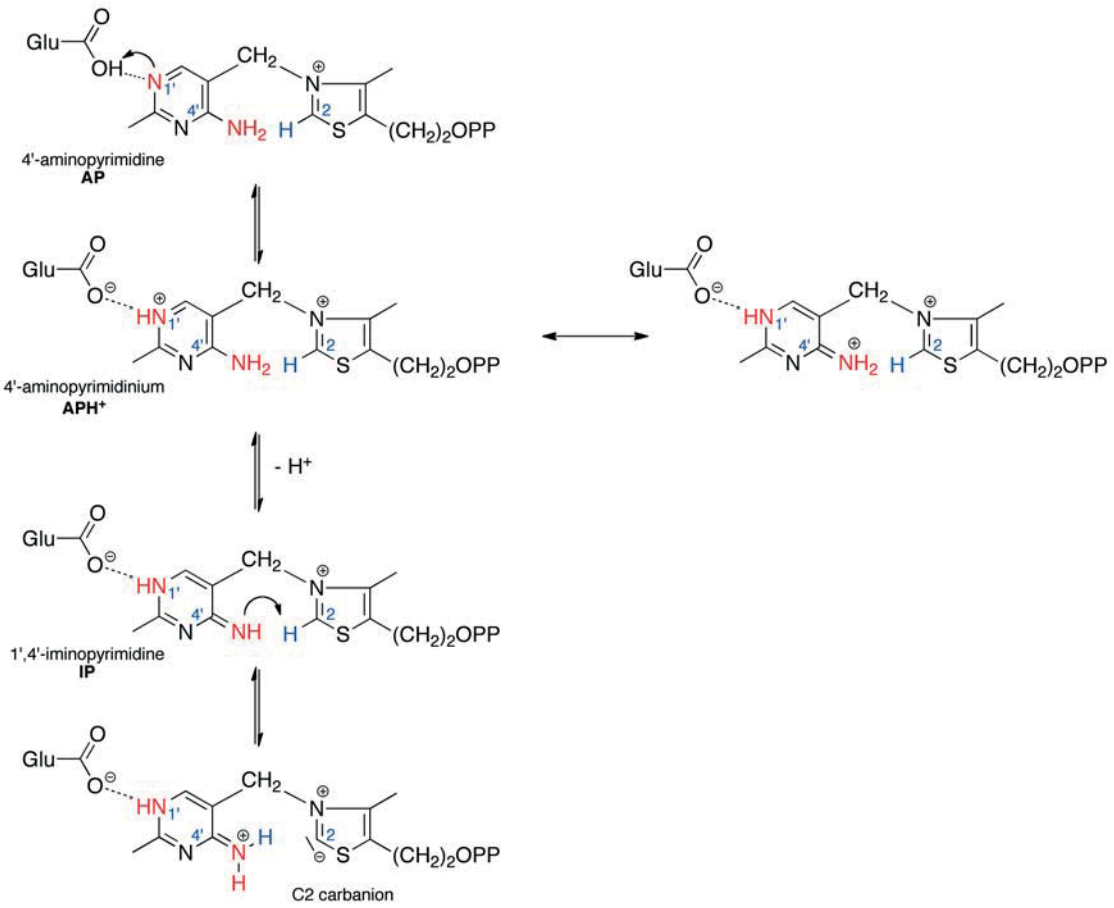

Figure 1.3: **Scheme illustrating activation of ThDP by conserved Glu-residue.** Picture taken and modified from Kluger and Tittmann [60].

In the first step the 1'-nitrogen atom of the pyrimidine ring is protonated by a highly conserved glutamate. This glutamate side chain, associated with protonation of 1'-nitrogen atom and subsequent deprotonation of C2, has widely been seen, in close proximity to the cofactor, in crystal

structures e.g. of PDC and transketolase (TK) (Kluger and Tittmann [60]). Replacement of the conserved Glu residue by other amino acids has been found to impair the ylide formation resulting in the loss of enzymatic activity. However, a recent study revealed that there is no such conserved Glu residue in the ThDP-dependent enzyme glyoxylate carboligase (GCL) and therefore the role of the Glu side chain seems not to be essential for thiamine catalysis (Kluger and Tittmann [60], Kaplun et al. [56]). After proton transfer from the conserved Glu, the ThDP cofactor tautomarizes from its 4′-aminopyrimidine (AP) first to the 4′-aminopyrimidinium (APH^+) form and after subtraction of a proton to the 1′-4′-iminopyrimidine (IP) form. Subsequently the imino-nitrogen acts as base and deprotonates the adjacent C2 generating the C2 carbanion/ylide. This is facilitated by the *V-conformation* that the cofactor assumes, placing the 4′-NH_2-group within interaction distance to the C2 reaction center (Pohl [88]).

The different protonations states of the pyrimidine ring (4′-aminopyrimidine (AP), 1′-4′-iminopyrimidine (IP) and 4′-aminopyrimidinium (APH^+)) can be spectroscopically discriminated as has been described in detail by *Nemeria & Jordan* and *Meyer & Tittmann* (Jordan et al. [53], Jordan [50], Nemeria et al. [81], Meyer et al. [75], Nemeria et al. [82], Nemeria et al. [84]).

By comparison of data from the native system of different enzymes with data generated with model compounds (e.g. ThDP-, intermediate- and substrate-analogues), the spectroscopic signatures in absorbance and CD spectroscopy were assigned for the AP- and IP-form. The AP-form was assigned to a negative CD band with a minimum at 320-330 nm and the IP-form assigned to a positive CD signal between 300-310 nm. So far, no assignment for the APH^+ form has been reported.

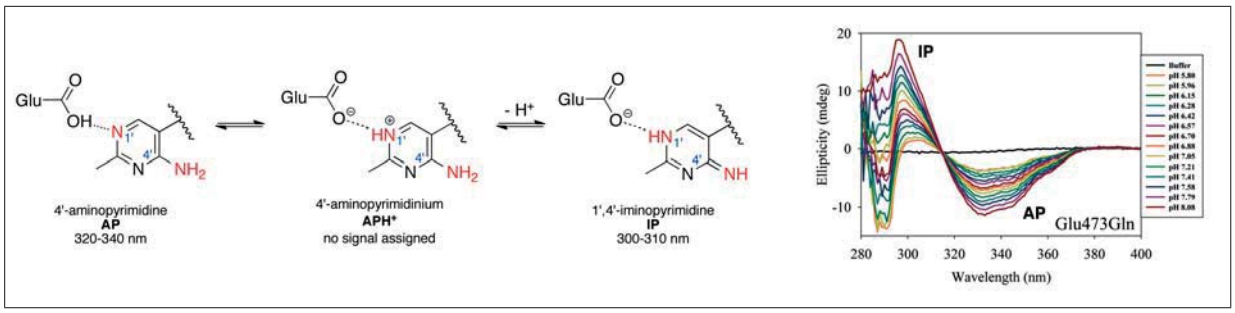

Figure 1.4: **Schematic illustration of protonation states of the aminopyrimidine ring of ThDP (left) and near-UV CD spectra of *Zm*PDC Glu473Gln with assigned bands (right).** Near-UV CD spectra taken and modified from Meyer et al. [75].

A simplified schematic illustration of the protonation states of ThDP with signature assignment is illustrated in fig.1.4. On the right is an illustration of the near-UV CD spectra of the *Zm*PDC variant Glu473Gln with the corresponding band assignment for the IP and AP form of the cofactor (see fig.1.4). *Meyer et al.* could clearly assign the different tautomeric states of the aminopyrimidine ring of the enzyme-bound ThDP. By pH titration they could prove with the examined pH dependency of the system that the shift in bands corresponds to the mechanism postulated for ThDP catalysis (Meyer et al. [75]).

1.4 Diversity of ThDP Catalysis

ThDP-dependent enzymes catalyze a broad variety of enzymatic reactions. They participate in numerous biosynthetic pathways and are mainly involved in the cleavage and formation of C–C bonds. For instance they catalyze the non-oxidative and oxidative decarboxylation of 2-keto acids, produce 2-hydroxy ketones and transfer activated aldehydes to a variety of acceptors. Moreover, they are able to catalyze C–N, C–O and C–S bond formation and cleavage. Due to their considerably wide substrate range and their intrinsic stereospecificity this class of enzymes is exploited for application in asymmetric syntheses (Schörken and G [99], Sprenger and Pohl [106], Pohl et al. [89], Samland and Sprenger [93], Müller et al. [79], Kurlemann et al. [67], Brovetto et al. [15]).

An overview of the promiscuous applicability of ThDP-dependent enzymes is given in fig. 1.5 gives, mainly on the basis of wild type enzymes (Müller et al. [79]):

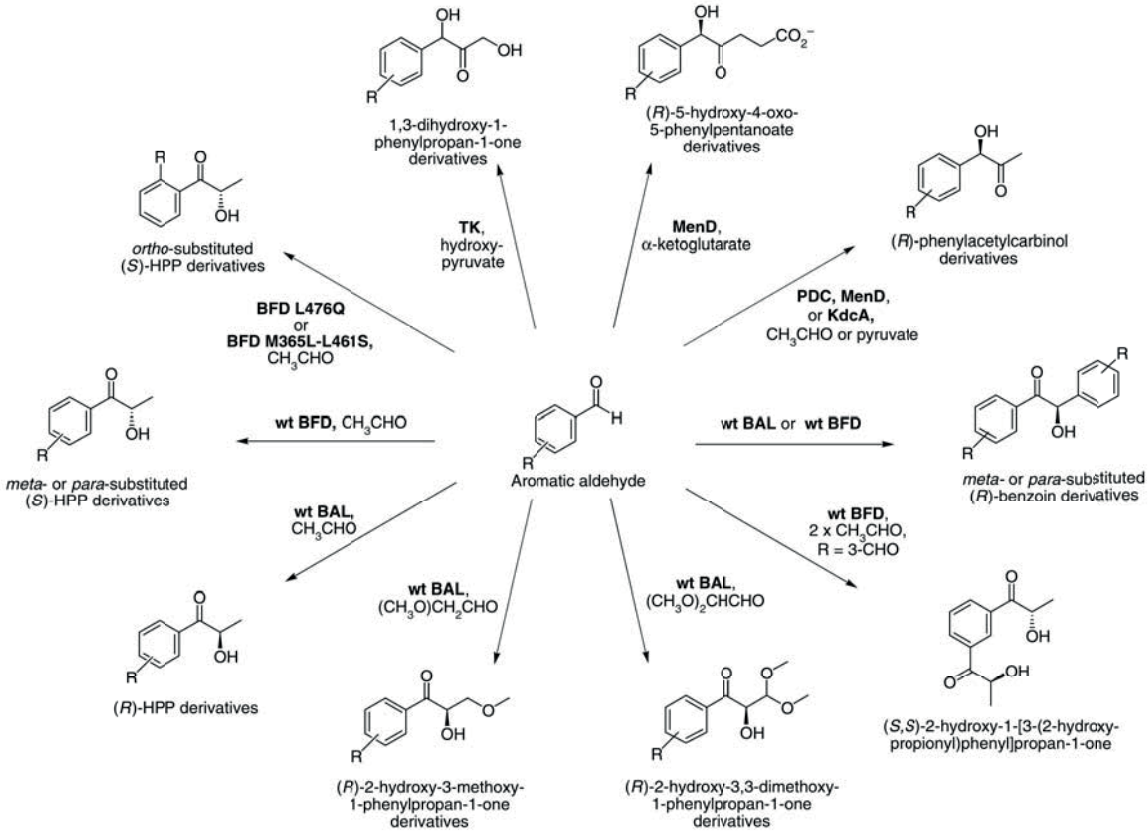

Figure 1.5: **Diversity of ThDP catalyzed enzymatic reactions.** Examples of ThDP-dependent enzyme-catalyzed transformations. (Picture taken from Müller et al. [79]).

Diverse C–C bond formations can be achieved by use of the depicted ThDP-dependent enzymes (see fig. 1.5). These enzymes have been widely applied onto synthetic organic transfor-

mations. Their successful application in asymmetric synthesis will be discussed in more detail with respect to PDC in chapter 1.7, and in a general context in chapter 1.9.

1.5 Pyruvate Decarboxylase – a ThDP-dependent Enzyme

General & Biological Relevance

Pyruvate decarboxylase (enzyme commission number (EC) 4.1.1.1) is a ThDP-dependent lyase, an enzyme that catalyzes bond construction and breaking by means other than hydrolysis or redox reactions (Brovetto et al. [15]) and additionally requires the cofactor ThDP for its catalytic activity.

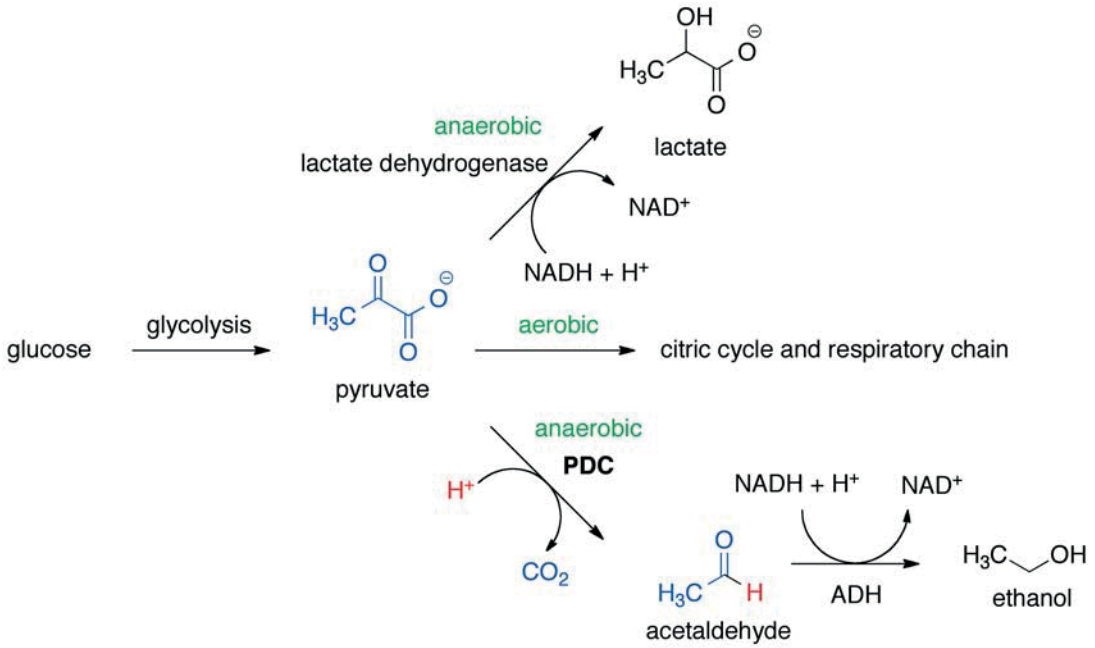

Figure 1.6: **Possible catabolization of glucose.** (Voet et al. [115])

PDC is the first enzyme of the branched glycolytic pathway that together with alcohol dehydrogenase (ADH) (EC 1.1.1.1) is responsible for the conversion of pyruvate into ethanol under anaerobic conditions (see fig.1.6). In detail, PDC catalyzes the decarboxylation of pyruvate into acetaldehyde and CO_2, the penultimate step in the non-oxidative part of the alcoholic fermentation. The enzyme requires the cofactor ThDP as well as Mg^{2+} for catalytic activity (Schellenberger [95]). During aerobiosis pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase (EC 1.2.4.1), while during anaerobiosis lactate or ethanol is formed to oxidize the NADH. (Voet et al. [115]).

Genes encoding for PDCs have been isolated from yeast or filamentous fungi such as *Saccharomyces cerevisiae*, *Hanseniaspora uvarium*, *Klyveromyces marxianus* and *lactis*, *Aspergillus parasiticus*, *Candida utilis*, *Neurospora crassa* and *Rhizopus javanicus* as well as from microorganisms

as *Zymomonas mobilis* and *Acetobacter pasteurianus*, and from plants such as maize (*Zea maize*), tomato (*Lycopersicon esculentum*), rice (*Oryza sativa*), pea (*Pisum sativum*) and tobacco (*Nicotiana tabacum*) (Brovetto et al. [15], Pohl [88]). PDCs from *S. cerevisiae* and *Z. mobilis* are the most intensively studied enzymes regarding their reaction mechanism and substrate specificity (of both the decarboxylase and carboligase reaction). Thus they are also the most widely used in organic synthesis (Brovetto et al. [15],Pohl [88], Iding et al. [48]).

In addition to its conventional reaction PDC also catalyzes a carboligation as minor side reaction (see fig. 1.7).

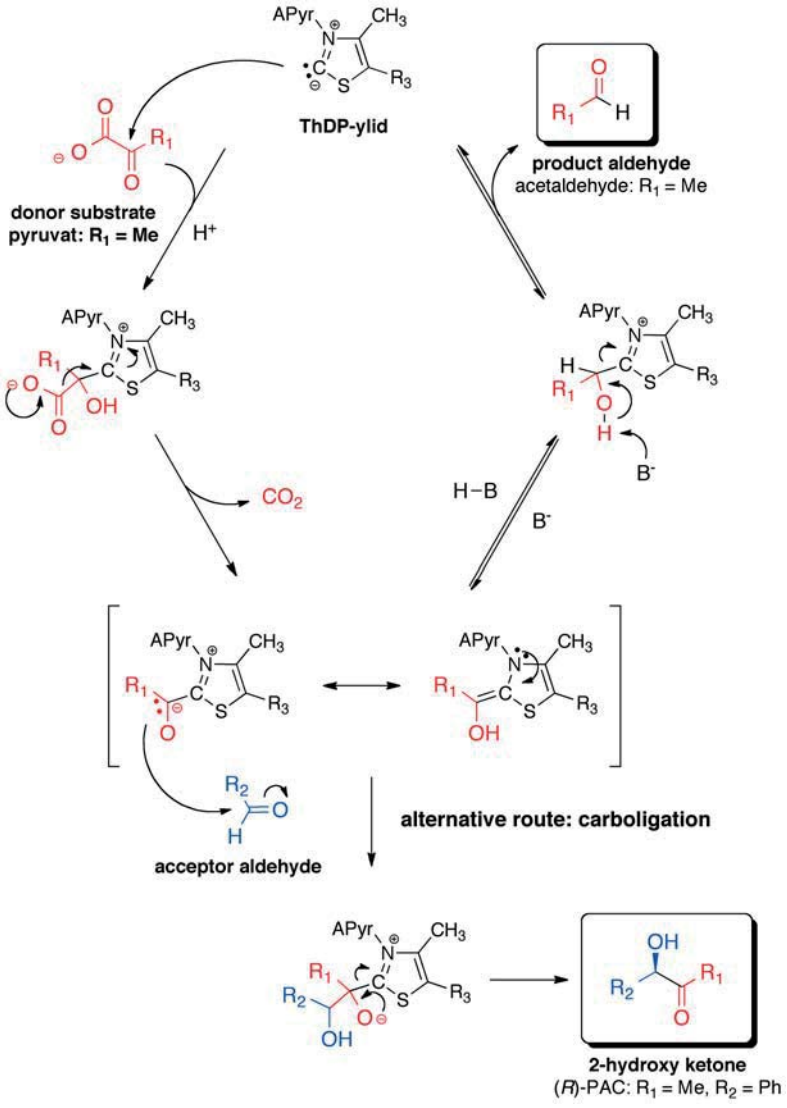

Figure 1.7: **General ThDP-mediated mechanism of native PDC catalysis with alternative carboligation route for 2-hydroxy ketone synthesis.** With the native substrate pyruvate the enzyme produces - after protonation - the innate product acetaldehyde. As alternative route the carboligation pathway is depicted: Here the carbanionic intermediate nucleophilically attacks an acceptor aldehyde and produces predominantly (*R*)-2-hydroxy ketones as e.g. (*R*)-phenylacetylcarbinol ((*R*)-PAC).

This formal acyloin condensation between an aldehyde and an adequate donor produces enantiomerically enriched (*R*)-2-hydroxy ketones. The key step in the catalytic mechanism is the reaction of the thiamine diphosphate cofactor with the donor substrate forming the active zwitterion. The attack of this active species to the acceptor aldehyde generates the (*R*)-2-hydroxy ketone upon releasing of the cofactor. With this promiscuous reactivity PDCs are exploited for the asymmetric synthesis of compounds like (*R*)-phenylacetylcarbinol and other 2-hydroxy ketones (Brovetto et al. [15]), which will be further elucidated in chapter1.7.

1.6 *Zm*PDC – A Prototypical ThDP-dependent Enzyme

1.6.1 *Zymomonas Mobilis*

Zymomonas mobilis, a gram-negative bacterium, is widely used for the fermentation of alcoholic beverages in tropic areas (Swings and De Ley [109]). Unlike fungi and plants *Z. mobilis* converts glucose or other sugars to pyruvate via the Entner-Doudoroff pathway. As this pathway exhibits low energy efficiency it requires the production of large quantities of ethanol (Conway [23], Gibbs and DeMoss [38]). Consequently, PDC is one of the most abundant proteins present in *Z. mobilis* (An et al. [3]). The Entner-Doudoroff pathway occurs only in procaryotes and describes a series of reactions that catabolize glucose to pyruvate. Nevertheless, most bacteria use glycolysis and the pentose-phosphate pathway for adenosine triphosphate (ATP) production (Conway [23]).

1.6.2 *Zm*PDC – Biochemical Properties

In contrast to PDC from yeast or plants bacterial PDCs, such as PDC from *Z. mobilis* (*Zm*PDC), are not subject to homotropic substrate activation. Yeast PDC for example requires the allosteric activation by binding a regulatory effector molecule, which in homotropic allosteric regulation is the substrate molecule of the enzyme (König [63], König et al. [64]). Hence, *Zm*PDC does not need activation through a substrate (pyruvate or surrogate pyruvamide) it is well-suited for kinetic measurements, simplifying the underlying kinetic model. It is a prototypical system for the investigation of the molecular reaction mechanism in the absence of additional complex conformational equilibria (Pohl [88], Meyer et al. [75]). In terms of its kinetic properties it exhibits a hyperbolic substrate dependence (v/S-plot) and is not influenced by phosphate, pyruvate or the surrogate pyruvamide, whereas all other PDCs exhibit a sigmoidal substrate dependence (v/S-plots) and a lag phase during product formation induced by their substrate activation (Pohl [88]). In addition, *Zm*PDC exhibits linear initial rates of product formation and hyperbolic dependence of initial rate on pyruvate concentration (Sun et al. [108]).

*Zm*PDC is a homotetramer with a molecular mass of 240 kDa. As in the other PDCs its activity is dependent on the cofactors ThDP and Mg^{2+}(Schellenberger [95], Diefenbach and Duggleby [25]). Even though Mg^{2+} is preferred by nature, it can be replaced by alternative bivalent cations