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Protein-enhanced decarboxylation of the covalent intermediate in benzoylformate decarboxylase—Desolvation or acid catalysis?

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This paper honors the memory of Miriam Hasson. Her work continues to inspire us.

Abstract

Benzoylformate decarboxylase (BFD) enhances the rate of decarboxylation of its key intermediate compared to the nonenzymic reaction by a factor of about 10^6. It has been proposed that desolvation into a hydrophobic environment will lower the reaction barrier in TDP-dependent decarboxylases. The competition of thiamin thiazolone diphosphate (TTDP) with the cofactor thiamin diphosphate (TDP) provides a dynamic indication of the relative hydrophobicity of the cofactor binding site. BFD binds the more polar TDP tightly in the presence of excess TTDP. Therefore, desolvation would not be likely to occur during catalysis. Unlike TDP enzymes that have electron acceptors as substrates, decarboxylases require protonation to produce the precursor to the aldehyde product. A mechanism involving an associated acid that traps the carbanion generated upon C–C bond breaking will permit diffusional separation of carbon dioxide and generate the appropriate precursor to the product aldehdye. This would also account for avoidance of a competitive reaction. Hasson's detailed structure of BFD shows a highly polar active site with histidines in the vicinity of the substrate. Reports of a reduction of k_{cat} to near the nonenzymic rate without a large effect on K_m upon specific replacement of these histidines with alanine fit this alternative. In TDP enzymes involving oxidation or condensation, an electrophilic substrate or second cofactor will be bound (and no proton will be required). This will acquire the electron density of the carbanion itself. In such

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cases, protonated side chains are not functional while hydrophobic environments would promote the internal transfer.

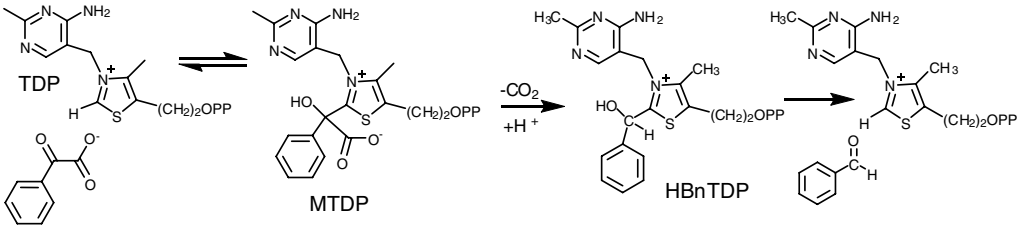
Keywords: Benzoyl formate decarboxylase; Thiamin thiazolone diphosphate; Catalysis of carbon dioxide separation; Decarboxylation; Protonation to form aldehyde precursor; Hydrophobicity; Histidines as acid catalysts; Contrasting reaction patterns; Aldehyde precursor; Proton donors versus Lewis acids

1. Introduction

Enzymes that utilize thiamin diphosphate (TDP) as a cofactor for the decarboxylation of 2-ketoacids produce covalent intermediates whose nonenzymic and enzymic reactivity can be measured and compared [1–13]. Interpretation of the resulting data provides a basis for general insights into the effects of a protein on specific steps of catalysis. Benzoylformate decarboxylase (BFD) presents a special opportunity for such an analysis as the enzyme's native and mutated structures are known at high resolution from the work of Miriam Hasson and coworkers [14–17]. The corresponding thiamin-derived intermediates (lacking the coenzyme's pyrophosphate group, which has no catalytic role) are available and their reaction rates and patterns have been determined [13,18,19].

The addition product from TDP and benzoylformate in BFD is 2-α-mandelyl-thiamin diphosphate (MTDP) (Scheme 1) [14]. The electronic structure of MTDP should encourage the spontaneous conversion of its carboxylate group to carbon dioxide, a process that leaves a pair of electrons at C2-α. Through delocalization, the negative charge is neutralized by the cationic immonium function of the thiazolium ring, increasing the acidity of the conjugate carbon acid at C2-α [20]. The immonium group functions as an internal Lewis acid catalyst for decarboxylation, usurping the role of any external acid catalyst in this regard [21,22]. After decarboxylation, the site from which carbon dioxide departs accepts a proton from water, producing the stable intermediate, 2-(1-hydroxybenzyl)TDP (HBnTDP).

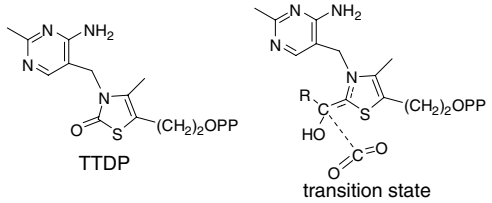
Based on the properties of this minimal mechanism, the carboxylate group of MTDP should be cleaved to form carbon dioxide with a rate constant that is at least as large as k_{cat} of BFD, since there is no role for an additional acid or base catalyst that the protein could provide. However, the reactivity of the close analogue of MTDP, 2-α-mandelyl-thiamin (MT), does not meet these expectations: k_{cat} for BFD is almost one million times larger than the first-order rate constant for decarboxylation of MT [13,18,23,24].



Scheme 1.

Furthermore, in the MT reaction, upon loss of carbon dioxide, protonation of the product to give 2-(1-hydroxbenzyl)thiamin (HBnT) competes with a rapid and destructive fragmentation of the thiamin core structure [25–27]. The fragmentation has a very low barrier: the first-order rate constant is greater than k_{cat} of BFD [12]. It is thus clear that this remarkable protein enhances the desired reaction and at the same time avoids an undesirable and inherently faster reaction of the product.

The means by which the decarboxylation step of TDP-conjugates of 2-ketoacids is accelerated has been the subject of speculation. Lienhard and coworkers observed that decarboxylation of an analogue of the pyruvate conjugate of thiamin is markedly accelerated in solvents that are less polar than water [28]. Nonpolar solvents also accelerate the decarboxylation of MT [13]. In the medium of lower polarity, the zwitterionic conjugate is destabilized (''stressed'' [29]) while the transition state is stabilized. Lienhard proposed that since enzymes function by binding transition states tightly, a stable molecule that resembles a partially transformed substrate should be bound especially tightly [30]. Since the transition state for decarboxylation is less polar than the reactant and decarboxylation to the delocalized carbanion involves rehybridization at C2α, Lienhard proposed that thiamin thiazolone diphosphate (TTDP) would be a transition state analogue inhibitor of TDP-dependent decarboxylases [31].



Gutowksi and Lienhard reported the predicted outcome with pyruvate dehydrogenase (PDH), an enzyme that forms an initial conjugate of TDP and pyruvate [31]. Later, the competition of TDP with TTDP in wheat pyruvate decarboxylase (PDC) was assessed with very different results [32]. The PDC reaction involves the same substrate and TDP-addition intermediate as does PDH but is associated with a different protein. In PDC, TTDP does not bind more tightly than does TDP [32]. Since the transition state structures in terms of the cofactor and pyruvate should be the same, this establishes that the tightness of binding of TTDP relative to TDP is more likely to be a measure of the hydrophobicity of the binding site. A similar conclusion was reached for the effects of TTDP with transketolase [33].

If the enhanced rate of decarboxylation of MTDP is the result of desolvation of the addition product at the active site of BFD, the cofactor binding site would be hydrophobic. The relative affinity of the protein for TDP and for TTDP can therefore be interpreted in terms of the reported structure and potential modes of catalysis. Thus, we have examined the interaction of BFD with TTDP and TDP.

2. Materials and methods

2.1. Synthesis

Thiamin thiazolone diphosphate was prepared according to the procedure of Gutowski and Lienhard [31].

2.2. Expression and purification of benzoylformate decarboxylase

This was done according to the method of Pohl and coworkers [34]. A spectrophotometric assay was used to obtain an approximate protein concentration [35]: protein concentration (mg/mL) = $1.55\ A_{280} - 0.76\ A_{260}$. An aliquot of the BFD preparation was then diluted and protein content was determined more accurately using the Bradford assay with bovine serum albumin as a standard [36]. The BFD enzyme was stored in 50 mM potassium phosphate buffer, pH 7.0 with 0.1 mg/mL of sodium azide as a preservative or at −20 °C as a lyophilisate.

2.3. Direct decarboxylase assay

The activity of the purified BFD–His_6 was determined by adding benzoylformate to the reaction mixture at 30 °C. The consumption of benzoylformate was followed at 343 nm for 40 min for three different amounts of BFD. The extinction coefficient at 343 nm for benzoylformate in 150 mM potassium phosphate, pH 7.0, containing 2.5 mM $MgSO_4$ was determined to be 79 $mM^{-1}\ cm^{-1}$. One activity unit is the amount of enzyme that converts 1 μmol of benzoylformate per minute under the above conditions.

2.4. Coupled decarboxylase assay

This was used for assaying the effects of TTDP. The components were incubated for 5 min at 30 °C. Three minutes prior to the addition of benzoylformate, HLADH (30 μL) and an aliquot of BFD were added to the reaction mixture. The reaction was followed by the decrease in absorbance of NADH at 340 nm over 60 min [37].

2.5. Effects of thiamin thiazolone diphosphate

BFD solution was added to the reaction mixture containing TTDP and/or TDP equilibrated at 30 °C for 5 min. The reaction was then started with the addition of benzoylformate and followed at 340 nm. For longer incubation times, the enzyme and TTDP were brought to a total volume of 300 μL and incubated at 5 °C for 13 min, 180 min, and 23 h. A 30 μL sample was then added to the reaction mixture and the absorbance at 340 nm was followed. See Tables 1 and 2 for conditions.

Table 1
Incubation mixtures of BFD and TTDP

Solution	Volumes (μL)				
BFD	100	100	100	100	100
Water	100	90	80	60	0
TTDP, 10 μM	0	10	20	40	100
[TTDP], μM	0	5	10	20	50

Table 2
Reaction mixture for study of time-dependence of addition of TTDP on BFD activity

Solution	Volume (μL)
Phosphate buffer, 400 nM, pH 7.0	100
$MgSO_4$, 40 mM	63
HLADH, 33 U/mL	50
NADH, 20 mM	20
Incubation mixture	30
Benzoylformate, 10 mM	37
Water	700
Total volume	1000

3. Results and discussion

3.1. Inhibition with thiamin thiazolone diphosphate

The effect of TTDP was studied with BFD in the presence of TDP. After 5 min incubation, activity was assayed for aliquots containing 0.075, 1.5, 2.2, and 3.0 μM TTDP (Fig. 1). In all cases, activity decreased over the next 30 min. Initially, the activity decreased somewhat more with higher concentrations of TTDP. Over longer incubation times this dependence was not maintained (Fig. 2). After further incubation of the holoenzyme with TTDP the results showed that TDP binds more effectively in this competition.

Removal of TDP from the enzyme required dialysis in 12 × 1 L of 50 mM phosphate buffer, pH 7.0 over one week. Alternatively, resuspension of cells in 12–15 mL lysis buffer/g wet cell weight preceding purification gave a BFD preparation without any activity. Although Iding et al. report that reconstitution of the apo-enzyme in 1 μM TDP gave half-maximal activity in 24 h [34], our attempts at reconstitution were not successful at any concentration of TDP, even with incubation for one week.

The addition of TTDP has no effect on the activity of BFD in the presence of TDP. Even high concentrations of TTDP, in significant excess of TDP, and long incubation periods (Table 1), do not affect the enzyme's activity relative to a control. This leads us to conclude that the enzyme's affinity for the active cofactor, TDP, is much higher than it is for

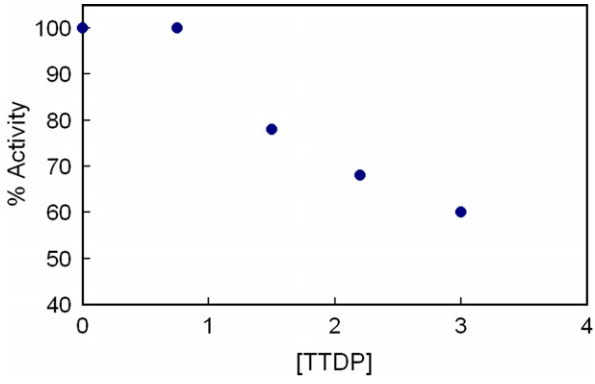


Fig. 1. BFD activity as a function of TTDP concentration after 5 min incubation.

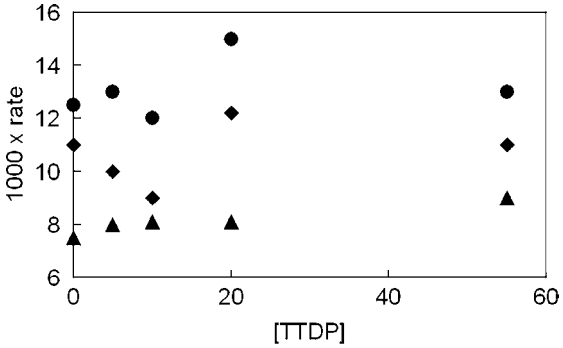


Fig. 2. Effect of TTDP on BFD activity over longer periods.

its hydrophobic analogue, TTDP (TDP binding is reversible $K_m = 1$ μM [34]). This establishes that TTDP is not a transition state analogue as it binds less tightly than TDP to BFD, nor is it competitive with TDP. Since TTDP is less polar than TDP, these results also are consistent with the cofactor's binding site on the enzyme not being hydrophobic. Thus, we conclude that since the protein does not utilize a hydrophobic cofactor-binding domain it does not accelerate the decarboxylation step by desolvation. This is consistent with the many polar functional groups seen in the active site of BFD in Hasson's X-ray structures of the native and mutated holoenzyme [14,15].

We have proposed that alternative mode of catalysis of decarboxylation of MTDP is achieved by preventing the addition of the product carbanion to nascent carbon dioxide. Protonation of the carbanion by an adjacent acid permits separation of the products and also blocks fragmentation. Such a mechanism accounts for the specific catalysis provided by the conjugate acid of pyridine and alkyl-pyridines in the decarboxylation of MT [24]. In the protein's active site, proton donors from nearby side chains would readily protonate the adjacent carbanion. Hasson's structure of BFD shows a number of Brønsted acids in the active site [14,16]. The effects of specific mutations that replace active site histidines with alanines are consistent with one or both of these groups being a participant in acid-promoted decarboxylation: In the H70A mutant, k_{cat} is reduced by a factor of more than 1000, while K_m for benzoylformate increases by only a factor of 5 [14]. This behavior is consistent with a major function of H70 being protonation of the carbanion resulting from decarboxylation since its primary involvement would be in enhancing catalytic efficiency. The mutant H281A has k_{cat} reduced by more than a factor of 100 compared to the wild type with little change in K_m of benzoylformate, which is also consistent with a catalytic function. Based on Hasson's structural analysis [14–16], His-70 and His-281 are sufficiently close to the substrate to transfer a proton to the carbanion formed upon breaking the carboxylate-carbon bond. From the observed rate parameters of the mutated proteins we see that are functional but the rate of decarboxylation of the intermediate could be as low as that of the nonenzymic model in the absence of added pyridine. Based on this hypothesis, in H70A, His-281 would serve as proton donor while in H281A, His-70 would serve as a proton donor. With both groups present, proton transfer could occur from either. The proton transfer will also suppress fragmentation since only the delocalized carbanion is subjected to fragmentation [13,18,24].

The kinetics of the reaction of an alternative substrate, *p*-nitrobenzoylformate have been measured at 620 and 420 nm, which were assigned to the absorbance of the p-nitro analogues of MTDP and of HBnTDP, respectively [14]. The enzyme does not succeed as well in accelerating the reaction of the nitro-substrate ($k_{cat} = 0.04\ s^{-1}$ and K_m is high). Electronic effects of the nitro substituent should enhance addition of TDP and decarboxylation of the intermediate, while protonation at C2α of the decarboxylation product would be slower due to its decreased basicity. This would make the enamine from HBnTDP more persistent and increase the possibility of reversing the reaction. The observed spectral decay curves and steady-state rate constants could be interpreted in these terms [24].

In conclusion, our data are consistent with a polar site in which TDP is bound. Transfer of a proton from imidazolium of histidine to the immediate product of decarboxylation derived from TDP and substrate will enhance the rate of decarboxylation by an amount that accounts for the enhanced rate of the enzymic reaction. At the same time, this suppresses the fragmentation reaction, which is prevented by protonation. Such a process is productive only where the ultimate product is an aldehyde, as in BFD and pyruvate decarboxylase [9]. However, such a mechanism cannot extend to enzymes where the carbanion is oxidized by an internal cofactor [38,39] (This is also exemplified by pyruvate oxidase [40] and pyruvate dehydrogenasae [31,41]) or in enzymes where the carbanion attacks a carbonyl carbon (as in transketolase [42]). In those cases, a hydrophobic environment may well be conducive to the proper reaction.

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