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A Bulky Hydrophobic Residue Is Not Required To Maintain the V-Conformation of Enzyme-Bound Thiamin Diphosphate

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Supporting Information

ABSTRACT: It is widely accepted that, in thiamin diphosphate (ThDP)-dependent enzymes, much of the rate acceleration is provided by the cofactor. *Inter alia*, the reactive conformation of ThDP, known as the V-conformation, has been attributed to the presence of a bulky hydrophobic residue located directly below the cofactor. Here we report the use of site-saturation mutagenesis to generate variants of this residue (Leu403) in benzoylformate decarboxylase. The observed 3 orders of magnitude range in k_{cat}/K_m values suggested that conformational changes in the cofactor could be influencing catalysis. However, X-ray structures of several variants were determined, and there was remarkably little change in ThDP conformation. Rather, it seemed that, once the V-conformation was attained, residue size and hydrophobicity were more important for enzyme activity.

The first step in catalysis by all ThDP-dependent enzymes is the deprotonation of C2 by the 4′-imino group to form the ylide (Figure 1A).[1−3] Subsequently, the substrate carbonyl is attacked by the ylide to form the tetrahedral ThDP−substrate adduct that then loses CO_2 to form the carbanion−enamine intermediate. For deprotonation to occur, the 4′-imino group must be within hydrogen bonding distance of the proton on C2. It is notable that, to date, all X-ray structures of ThDP-dependent enzymes have shown that the two groups are within 3.5 Å, well within the requisite distance for deprotonation.[4−8] Further, the structures suggest that it is the buckling of the aromatic rings of ThDP around a bulky hydrophobic residue, generally leucine, isoleucine, or methionine and, on rare occasions, phenylalanine, that brings the two groups together (Figure 1B).[8] This conformation of ThDP, often termed the V-conformation, is rarely observed when free in solution[3] but is the only conformation that has been observed for enzyme-bound ThDP.

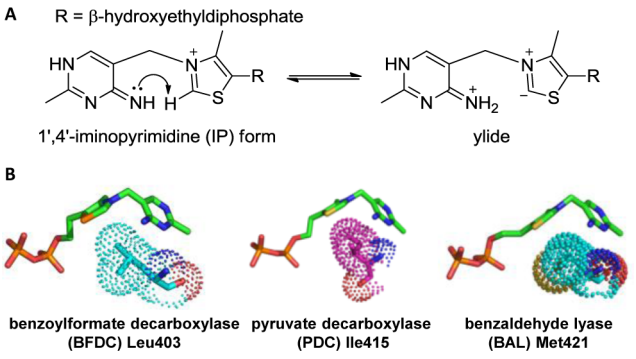

Figure 1. (A) Deprotonation of C2 forming the reactive ylide. (B) Representative V-conformations of ThDP assisted by leucine, isoleucine, and methionine.

In benzoylformate decarboxylase (BFDC), the V-conformation is maintained by a leucine residue, Leu403 (Figure 1B), and results in a N4′−C2 distance of 3.1 Å.[6] It has been predicted that the fundamental attributes of the V-conformation will be preserved regardless of the identity of the residue at position 403 and that, for each enzyme, the bulky residue that best fits the backbone will have evolved.[8] Recently, our group used site-saturation mutagenesis to examine residues in benzoylformate decarboxylase (BFDC) thought to be involved in catalysis[9] and substrate specificity.[10] The results proved to be unexpected and necessitated a re-evaluation of the entire mechanism.[9] Given those results, it seemed saturation mutagenesis could also be used to examine whether (i) a bulky hydrophobic residue is required to maintain the V-conformation of ThDP and (ii) each ThDP-dependent enzyme's active site has evolved to accommodate a specific hydrophobic residue.

The saturation mutagenesis of Leu403 and subsequent activity screening were conducted as described for earlier mechanistic studies.[9] Unexpectedly, more than half the colonies screened had significant (>10%) wild-type (wt) activity. Plasmids carrying unique active mutations as identified by sequencing and determined to be active by the plate reader screen were used for expression, purification, and kinetic characterization of several L403X variants. The 12 variants purified were shown to be active, but with one exception, all mutations resulted in a decrease in k_{cat} values (Table 1). Replacing leucine with isoleucine, a residue widely used by other decarboxylases at this position, had no effect on k_{cat} and produced an only modest 2-fold increase in K_m. By contrast, the analogous variant of PDC (I415L) showed a 12-fold decrease

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Table 1. Kinetic Constants for BFDC L403X Variants[a]

variant	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
wt	390 ± 30 (1)	0.27 ± 0.02 (1)	1450 (1)
L403I	400 ± 35 (1)	0.56 ± 0.09 (2)	710 (2)
L403V	120 ± 3 (3.3)	0.56 ± 0.07 (2)	210 (7)
L403T	200 ± 40 (2)	1.1 ± 0.2 (4)	180 (8)
L403M	87 ± 10 (4.5)	0.7 ± 0.1 (2.5)	120 (12)
L403F	100 ± 2 (4)	2.1 ± 0.2 (8)	48 (30)
L403Y	not determined	not determined	23 (70)
L403E	22 ± 3 (18)	1.4 ± 0.2 (5)	16 (90)
L403C	7 ± 0.7 (56)	0.5 ± 0.1 (2)	14 (110)
L403Q	23 ± 1 (17)	1.8 ± 0.2 (7)	13 (115)
L403S	5.6 ± 0.2 (70)	0.7 ± 0.03 (2.5)	8 (180)
L403N	5.7 ± 0.4 (70)	2.4 ± 0.6 (9)	2 (725)
L403K	4.1 ± 0.4 (95)	3.1 ± 0.4 (12)	1 (1450)

[a]Values are means of at least three independent determinations ± the standard error. In parentheses are the *x*-fold changes vs the wt value.

in k_{cat}.[8] It was also found that leucine could be replaced with either the shorter hydrophobic residue valine or the more polar threonine, with similar effects on kinetic constants. Substituting with methionine, the other residue widely used by ThDP-dependent enzymes, resulted in a 1 order of magnitude decrease in k_{cat}/K_m, again an effect much weaker than that observed for PDC.[8] Perhaps more surprisingly, leucine could be replaced with either the negatively charged glutamate or the positively charged lysine with only ∼20- or ∼100-fold decreases in k_{cat} values, respectively.

With the exception of L403N and L403K, K_m values were relatively unaffected. That said, the aromatic variants L403F and L403Y did exhibit increased K_m values, such that the activity of the latter could be determined only under V/K conditions. The L403W variant did not express, so it was impossible to determine if this trend continued.

In addition to the kinetic analyses, seven of these variants were successfully crystallized and their X-ray structures determined. The resolution of the structures ranged from 1.07 to 1.39 Å (Table 2). The most immediate and striking

Table 2. Bond Distances (angstroms) and Angles (degrees) for L403X Variants

variant	PDB entry[a]	N1′−E47	N4′−C2	V_{angle}
wt	1BFD (1.60)	2.71	3.08	115.0
wt (TZD)	1YNO (1.22)	2.67	3.17	112.7
L403I	4GM4 (1.29)	2.72	2.97	114.1
L403T	4GG1 (1.07)	2.68	3.20	116.0
L403M	4GPE (1.39)	2.68	3.22	115.8
L403F	4GP9 (1.07)	2.71	3.40	118.8
L403E	4JD5 (1.33)	2.67	3.17	119.9
L403N	4GM0 (1.07)	2.68	3.22	114.8
L403S	4GM1 (1.26)	2.65	2.87	114.0

[a]In parentheses is the resolution of the structure in angstroms.

observation was that, as predicted, there was very little change in the V-conformation of the cofactor or, indeed, the enzyme as a whole. It was also clear that thiamin thiazolone diphosphate (TZD) with partial occupancy of the C2 oxygen was a better fit to the electron density than ThDP. While unexpected, this is not unprecedented; there have been previous reports of the presence of TZD bound by ThDP-dependent enzymes during structure determination. Generally, this has simply been attributed to radiation damage,[11] although hyperactivity of the C2 atom with molecular oxygen has also been reported to result in TZD formation.[12] Fortunately, the structure of the BFDC−TZD complex had been determined [Protein Data Bank (PDB) entry 1YNO]. Therefore, we were able to directly compare the geometry of TZD when it was bound by wt and L403 variants (Figure 2).

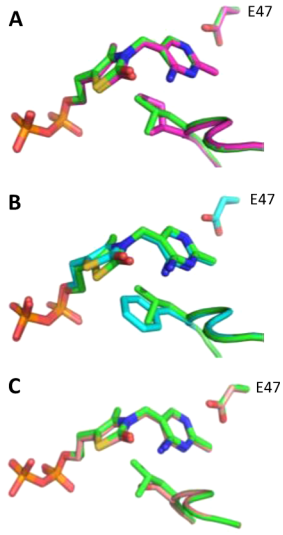

Figure 2. Overlay of the structures of (A) wt and L403I, (B) wt and L403F, and (C) wt and L403S. Shown are TZD, Glu47, and L403X. Structures were obtained by backbone superimposition, and in each case, the wt is colored green.

Even though the overall cofactor conformation is relatively unaffected, there is still some evidence of adaptation to the protein environment. For example, in the L403I variant, the isoleucine side chain has rotated so that the methyl groups of isoleucine occupy the same space as the γ-methyl groups of leucine (Figure 2A). As a result, the torsion angles of the cofactor, and the kinetic constants, are virtually identical to those of the wt enzyme.

The L403F variant is the only variant that substantially displaces the cofactor (Figure 2B), pushing the thiazolium ring away so that the root-mean-square deviation between the cofactors is 0.98 Å (compared with an average of 0.2 Å in the other variants). Consistent with the increase in K_m values shown by the aromatic variants (Table 1), there is also some movement (not shown) in Thr377, a residue known to be involved in substrate specificity.[10] To date, X-ray structures have been reported for only two ThDP-dependent enzymes using a phenylalanine residue at this position.[13,14] Pyruvate-ferredoxin oxidoreductase (PFOR, PDB entry 2C42) and carboxyethyl arginine synthase (CEAS, PDB entry 2IHT) both bind ThDP in a V-conformation, but the torsion angles between the pyrimidine and thiazolium rings (V_{angle}) of these two enzymes, ∼110° and ∼100°, respectively, are distinctly different from one another and smaller than those observed in other ThDP-dependent enzymes. It is notable that the L403F variant of BFDC shows neither the geometry of ThDP nor the rotamer of phenylalanine observed in PFOR or CEAS.

In some ways, the most intriguing structure was that of the L403S variant. While the k_{cat} values of L403S and L403I differed by more than 2 orders of magnitude, there were no apparent differences in the *B* factors (not shown) or TZD

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conformations (Figure 2C). Clearly, the much smaller serine residue is just as capable of maintaining the V-conformation as leucine or isoleucine, and it can do this without making any contact with the cofactor. It would appear that, consistent with earlier predictions,[8] the hydrogen bonds to the diphosphate tail and the aminopyrimidine ring, as well as the interactions with Mg^{2+}, are sufficient to constrain the cofactor. Given that these types of interactions are common to the members of this family, it is reasonable to assume that this result will be found for all ThDP-dependent enzymes.

In spite of their structural similarity, the variants exhibit distinct differences in catalytic constants that bear examination. In search of a ready explanation, we turned to two factors that play an essential role in the mechanism of ThDP-dependent enzymes.

The first is the hydrogen bond between N1′ and E47 that helps stabilize the imino tautomer, facilitating the formation of the ylide (Figure 1A).[15] As shown in Table 2, these distances were within 0.06 Å for all variants, a result that highlights the similarity of the positions of the cofactors within the active site and suggests that there will be little or no difference in tautomerization. Additionally, the formation of the ylide requires that the 4′-imino group be within H-bonding distance of C2. For TZD on the wt enzyme, that distance is 3.17 Å. In the variants examined here, the distance ranges from 2.87 Å (L403S) to 3.40 Å (L403F) (Table 2). Given that range, it is unlikely that the minor deviation (0.1 Å) in the N4′−C2 distance between the L403I and L403S variants is responsible for sufficient difference in rates of deprotonation of C2 to account for their significant disparity in k_{cat} values.

Attempts were also made to correlate the changes in activity with factors such as the surface area or volume of the various side chains. Arguably, if only the aromatic and hydrophobic residues were counted, a relationship between surface area and k_{cat}/K_m is observed, with an optimal surface area of ∼170 Å^2 (Table S1 of the Supporting Information). Unfortunately, the relationship breaks down when polar residues are included, and there is no obvious relationship with the volume of the side chain (Table S2 of the Supporting Information). The rate-determining step for the BFDC reaction is the addition of the ylide to the carbonyl of benzoylformate, with decarboxylation and product release being at least 1 order of magnitude faster.[16] However, the rate of C2 deprotonation has not yet been determined. Without knowing which step is affected in an individual L403 variant, we find it difficult to rationalize the observed rate changes.

Taken together, our data provide sufficient experimental evidence to predict that, on any ThDP-dependent enzyme, the V-conformation of the cofactor will be seen regardless of the identity of the "fulcrum" residue. Further, while a hydrophobic residue is preferred, it is not essential. Finally, it is also clear that BFDC is more tolerant of substitutions than yeast PDC,[8] so the question of whether individual enzymes have evolved specific residues for that position remains open.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and diffraction data collection and refinement statistics. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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