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A STUDY ON KINETIC CHARACTERIZATION OF CHANNEL-BLOCKING MUTANTS IN BRADYRHIZOBIUM JAPONICUM UTILIZATION A

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ABSTRACT Proline utilization A (PutA) from Bradyrhizobium japonicum (BjPutA) is a bifunctional flavoenzyme that catalyzes the oxidation of proline to glutamate using fused proline dehydrogenase (PRODH) and Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH) domains. Recent crystal structures and kinetic data suggest an intramolecular channel connects the two active sites, promoting substrate channeling of the intermediate P5C from the PRODH domain to the P5CDH domain. In this work several mutations were made along the channel in an effort to block passage of P5C to the second active site. Analysis of several site-specific mutants in the substrate channel of BjPutA revealed an important role for D779 in the channeling path. BjPutA mutants D779Y and D779W significantly decreased the overall PRODH-P5CDH channeling reaction indicating that bulky mutations at residue D779 impede travel of P5C through the channel. Interestingly, D779Y and D779W also exhibited lower P5CDH activity, suggesting that exogenous P5C must enter the channel upstream of D779. Replacing D779 with a smaller residue (D779A) had no effect on the catalytic and channeling properties of BjPutA showing that the carboxylate group of D779 is not essential for channeling. An identical mutation at D778 (D778Y) did not impact BjPutA channeling activity. Thus, D779 is optimally orientated so that replacement with the larger side chains of Tyr/Trp blocks P5C movment through the channel. The kinetic data reveal not only that bulky mutations at residue D779 hinder passage of P5C to the second active site, but also P5C must use the channel to efficiently access the P5CDH domain. Moreover, these mutants may be used to learn more about the hydrolysis event that is thought to take place within the channel.

KEYWORDS : Proline utilization A, Kinetic Characterization, Bradyrhizobium japonicum.

INTRODUCTION

Proline utilization A (PutA) catalyzes the conversion of proline to glutamate using two consecutive reactions. In the first step, proline dehydrogenase (PRODH) uses a flavin cofactor as an electron acceptor to remove two electrons from proline, resulting in Δ1-pyrroline-5-carboxylate (P5C). P5C then undergoes a non-enzymatic hydrolysis, which opens the pyrroline ring to create glutamate-γ-semialdehyde (GSA).

GSA is a substrate for NAD+-dependent pyrroline-5-carboxylate dehydrogenase (P5CDH), where two additional electrons are removed generating glutamate. Proline and proline metabolism are important to all walks of life. For example, proline plays important roles in different pathogens including pathogenicity of Helicobacter pylori and H. hepaticus, energy production for procyclic trypanosomes, and regulation of metabolites linked to pathogenesis in Photorhabdus and Xenorhabdus. In humans, inborn errors in PRODH are linked to schizophrenia. PRODH is also regulated by p53 and has been shown to suppress cancer [1].

PutA occurs as a bifunctional enzyme in all Gram-negative bacteria and Corynebacterium [2]. A recent crystal structure of Bradyrhizobium japonicum PutA (BjPutA) revealed a tunnel connecting the two active sites[3]. Structural and kinetic results suggest P5C/GSA uses the channel to travel from the PRODH active site to the P5CDH active site, without equilibrating with external solvent in a phenomenon called substrate channeling[3]. Three different mechanisms of substrate channeling have been identified in nature. The classic example includes an intramolecular channel connecting two active sites. Tryptophan synthase remains the most studied enzyme using this method of channeling. A second mechanism for channeling involves the intermediate moving across the surface of the enzyme. Two variations of surface channeling have been reported: dihydrofolate reductase-thymidylate synthase, which uses a surface “electrostatic highway”, and dethiobiotin synthetase- diaminopelargonic acid aminotransferase, which uses a polar crevice along the enzyme's surface [4]. A third mechanism of substrate channeling involves using a swinging arm to transfer intermediate to multiple sites. The pyruvate dehydrogenase complex provides an example where a lipoic acid cofactor acts as an arm [5].

Several physiological benefits of substrate channeling versus free diffusion have been identified. Chiefly, channeling decreases transit time between active sites and prevents loss of intermediates by diffusion, making the overall reaction more efficient. Thus, channeling enzymes can operate at maximum rates even if cellular substrate concentrations are not at saturating levels. Also, labile intermediates can be concealed from solvent to prevent decay or interaction with other molecules. Finally, reaction intermediates from competing reactions can be kept separate, which dictates metabolic flux [6].

As mentioned, a crystal structure of BjPutA revealed a tunnel connecting two active sites, which is thought to channel intermediate P5C/GSA. Theoretically, the channeling of P5C/GSA is physiologically reasonable, as P5C is a substrate of three competing reactions. It can be converted to glutamate via P5CDH, ornithine via ornithine aminotransferase, or back to proline via P5C reductase. Thus, channeling of P5C in proline catabolism may be necessary to retain proper metabolic flux and avoid metabolic cycling [7]. Besides enzyme competition, free P5C/GSA is reported to be an inhibitor of glutamine in three different E. coli enzymes including glucosamine-6-phosphate synthase, cytidine 5'-triphosphate synthase, and the amidotransferase domain of carbamoyl phosphate synthetase. Additionally, P5C was shown to form adducts with other important metabolic intermediates including oxaloacetic acid, pyruvic acid, and acetoacetic acid [8]. Considering the outcomes of liberated P5C/GSA, there appears to be a physiological advantage to sequester the intermediate during proline catabolism.

Substrate channeling in PutA was first kinetically described in Salmonella typhimurium; however, the structural and kinetic characterization of BjPutA have made this enzyme the prototype of PutA channeling. An X-ray structure of BjPutA revealed an irregularly-shaped cavity that spans 41 Å between the two active sites with a total volume of 1400 Å3 [3]. The majority of the volume (1325 Å3) of the tunnel comes from a large central chamber with dimensions 24 Å by 14 Å by 3-7 Å, which provides adequate space to accommodate P5C (102 Å3) or GSA (120 Å3) [2].

Additionally, kinetic evidence comparing native BjPutA to an equal molar mixture of two complementary active site mutants (R456M, no

PRODH activity; C792A, no P5CDH activity) showed the native enzyme immediately formed NADH, while the mixed variant system had a significant lag phase before reaching steady state [3]. Taken altogether, substrate channeling of P5C/GSA in BjPutA is supported by strong kinetic and structural evidence.

Using the structural and kinetic knowledge of the channel, this work further explores the cavity by mutating residues along the channel in an effort to obstruct P5C movement between the active sites. A combination of kinetic and structural results (through personal correspondence with Dr. John Tanner) provide evidence that channeling can be hindered, but the location of the mutation along the channel seems to be critical for impeding the intermediate. By making mutations along different parts of the channel, we further define the channeling path and provide novel insights into how P5C enters the channeling cavity.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich or Fisher Scientific unless otherwise noted. E. coli strain BL21 (DE3) pLysS was purchased from Novagen, and DH5α strain was purchased from Invitrogen. All experiments used Nanopure water.

Expression And Purification Of BjPutA

PutA from Bradyrhizobium japonicum (BjPutA) was expressed as reported previously, only the incubator temperature was decreased to 20 °C for 16 hours following IPTG induction. Cells were harvested by centrifugation and frozen at -80°C.

Frozen cells were re-suspended in 50 ml binding buffer (20 mM Tris-base, 0.5 M NaCl, 5 mM imidazole, 10% glycerol, pH 7.9) and 100 μM flavin at 4°C. Protease inhibitors ε- amino-N-caproic acid (3 mM), phenylmethylsulfonyl fluoride (0.3 mM), leupeptin (1.2 μM), tosyl phenylalanyl chloromethyl ketone (48 μM), and tosyllysine chloromethyl ketone hydrochloride (78 μM) were added, and cells were disrupted via sonication. The cell lysate was centrifuged for 1 h at 19,000 rpm in a JA-20 rotor (Beckman) and filtered through a 0.2 μm filter (VWR). Cell-free lysate was loaded onto a Ni-NTA Superflow resin (Qiagen) pre-equilibrated with binding buffer. Wash buffer (60 mM imidazole) then elution buffer (500 mM imidazole) were applied to the column. Protein in the elution fractions was then dialyzed into buffer containing 50 mM Tris (pH 7.5), 10 mM NaCl, 0.5 mM EDTA, 10% glycerol and loaded onto an anion exchange column (HiTrap Q HP column, GE Life Sciences) equilibrated with dialysis buffer (50 mM Tris, pH 7.5, 10 mM NaCl, 0.5 mM EDTA, 10% glycerol). A linear salt gradient comprised of dialysis buffer and dialysis buffer containing 1 M NaCl was applied to elute the PutA proteins. Purified PutA enzymes were then dialyzed into a final buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM Tris(3-hydroxypropyl) phosphine, and 10 % glycerol. Enzyme concentrations were determined using 660 nm Protein Assay (Pierce) and the amount of bound flavin was quantified (ε=13,620 M-1 cm-1) (27). The concentrations of the PutA proteins were normalized to the concentration of bound flavin, and the protein was flash-frozen in liquid nitrogen and stored at -80 °C.

Site-Directed Mutagenesis

Mutagenic primers (Table 1) were purchased from Integrated DNA Technologies or Eurofins MWG Operon. GeneTailor Mutagenesis Kit (Invitrogen) was used to generate all mutants except T348Y, which used a Quickchange II kit (Stratagene). Mutant plasmids were transformed into DH5α cells, and resulting plasmids were sequenced by.

Table 1. Primers Used For Site-directed Mutagenesis

Mutant	Primers
T348Y	5'GCGCCTATTGGGACTACGAGATCAAGCGCGCG 3'
	5' CGCGCGCTTGATCTCGTAGTCCCAATAGGCGC 3'
S607Y	5' AGACGCTCGACGATGCGCTCTATGAGCTGCGCG 3'
	5' GAGCGCATCGTCGAGCGTCTTGCCGCCCTCG 3'
D778Y	
	5'GCTGCCGGAGCAGGTCGCCTACGACGTTGTCACC3'
	5' GGCGACCTGCTCCGGCAGCGCGGTGGCATCG 3'
D779A	
	5'TGCCGGAGCAGGTCGCCGACGCCGTTGTCACCTCC 3'
	5' GTCGGCGACCTGCTCCGGCAGCGCGGTGGC 3'
D779W	
	5' TGCCGGAGCAGGTCGCCGACTGGGTTGTCACCTCC 3'
	5' GTCGGCGACCTGCTCCGGCAGCGCGGTGGC 3'
D779Y	
	5' CCGGAGCAGGTCGCCGACTACGTTGTCACCTCCGC 3'
	5' GCGGAGGTGACAACGTAGTCGGCGACCTGCTCCGG 3'

Channeling Assays

Channeling assays that monitor the coupled PRODH-P5CDH reaction were used to detect a lag phase in NADH formation when using proline as a substrate [3]. A lag in NADH formation indicates substrate channeling is not occurring. Channeling assays were performed at 23°C by following NAD+ reduction at 340 nm (ε = 6200 M-1 cm-1) as previously reported [3]. Assay conditions and spectral corrections were carried out identically to what was reported previously[3]. All assays contained 0.1 mM CoQ1, 0.2mM NAD+, 40 mM proline, 50 mM potassium phosphate (pH 7.5), 25 mM NaCl, 10 mM MgCl2, and 0.5 μM PutA enzyme.

Additional channeling assays were performed using increased concentrations of D779Y and D779W. Assays contained 0.1 mM CoQ1, 0.2 mM NAD+, 40 mM proline, 50 mM potassium phosphate (pH 7.5), 25 mM NaCl, and 10 mM MgCl2. Reaction progress curves were followed using a Cary Eclipse fluorescence spectrophotometer. NADH formation was monitored by exciting at 340 nm and recording the emission at 460 nm.

D779Y and D778W concentrations of 0.187, 0.374, 0.935, and 1.87 μM were used in the assays and compared to assays using 0.187 μM wild-type BjPutA.

Single Turnover Rapid-reaction Kinetics

Single turnover experiments were performed as described previously [3]. Briefly equal volumes of enzyme (21.3 μM wild-type, 17.8 μM T348Y, and 17.9 μM D779Y) with 50 mM potassium phosphate (pH 7.5), 25 mM NaCl, 0.1 mM NAD+ was rapidly mixed with 40 mM proline in 50 mM potassium phosphate buffer (pH 7.5), and 25 mM NaCl under anaerobic conditions (all concentrations reported as final concentrations after mixing)[9]. Anaerobic conditions were achieved by degassing buffer, substrate, and enzyme solutions by performing repeated cyles of nitrogen flushing and vacuuming. A day prior to performing the experiment, degassed buffer was imported into an anerobic glovebox (Belle Technology), where it was combined with protocatechuate dioxgenase (PCD) (0.05 U/mL) and protocatechuic acid (PCA) (100μM), which help scrub dissolved oxygen. Buffer was then loaded into syringes and sealed with parafilm before exporting from glove box. Stopped-flow mixing cell and tubing was thoroughly washed and incubated overnight with PCA/PCD buffer. Immediately prior to performing the experiment, enzyme and substrate were degassed and imported into the chamber, where PCA and PCD were added at concentrations reported above. Both enzyme and substrate were loaded into syringes and wrapped with parafilm before leaving the chamber. To collect data, anaerobic solutions were quickly loaded onto the stopped flow and syringes were parafilmed. Spectra from 300-700 nm were recorded using a Hi-Tech Scientific SF-61DX2 stopped flow instrument equipped with a photodiode array detector. FAD (451 nm) and NAD+ (340 nm) reduction reactions were extracted from the spectral traces and observed rate constants were determined by single exponential fits as previously reported [3].

Kinetic Parameters Of Alternative Substrates

Alternative P5CDH substrates were used to determine whether BjPutA mutants D779Y and D779W exhibit higher activity with smaller substrates relative to larger substrates. Kinetic assays using GSA, succinate semialdehyde, and propionaldehyde were performed, and Km and kcat values for each were determined. All assays were performed in buffer composed of 50 mM potassium phosphate (pH 7.5, 25 mM NaCl) containing 0.2 mM NAD+ and variable concentrations of GSA (L-P5C 0.01-6 mM), succinate semialdehyde (0.05-20 mM), and propionaldehyde (5-500 mM). Wild-type, D779A, D779Y, and D779W concentrations varied in the assays for each of the substrates. With GSA/P5C, all enzyme concentrations were 0.25 μM in the assays. For succinate semialdehyde wild-type and D779A were 0.25 μM while D779W and D779Y were 1 μM. For propionaldehyde, wild-type and D779A were 0.25 μM, D779W was 1μM, and D779W was 2 μM. All initial velocity data was collected on a Power wave XS 96 well plate reader (Biotek). The data were fit to the Michaelis-Menten equation using SigmaPlot 12.0.

RESULTS AND DISCUSSION

Six mutations were made at three different points along the putative

substrate channel of BjPutA. Of the six mutations made, only D779Y and D779W appear to disrupt P5CDH activity. Further kinetic results show that the PRODH and P5CDH domains are folded properly, which is also confirmed by X-ray crystal structures of both mutants (Tanner, personal communication). Finally, using smaller P5CDH domain substrates appears to increase the efficiency of the P5CDH active site relative to wild- type, especially for the more bulky D779W mutation. The results of this study verify that the cavity connecting the two active sites is used to shuttle P5C from the PRODH site to the P5CDH site, and that blockage of the channel decreases P5CDH activity.

Three separate mutations were made at D779 that provide details about the channel. First, mutating the aspartic acid to tyrosine dramatically decreases the catalytic efficiency of GSA by over 80-fold. Adding more bulk in the form of a tryptophan decreases the catalytic efficiency by over 940-fold, suggesting that larger residues at this position have a great effect on channeling P5C/GSA. Conversely, the alanine mutant at the same position displays similar kinetics at both active sites and channels similarly to wild-type. Additionally, results with D779A show that loss of the carboxyl group did not impact channeling, indicating charge at this location is not critical.

It is interesting to note that the position of the mutation along the channel is very important for channel disruption. D778Y is adjacent to D779Y/W and crystal structures show it slightly orients into the cavity, though not enough to constrict the channel. Consistent with the structural predictions, the D778 mutant channels similarly, if not slightly better than wild-type. Additionally T348 and S607 appear to point into bottleneck regions of the channel, but the tyrosine mutations do not affect activity at either active site or the overall channeling reaction. In terms of T348Y and S607Y, we cannot rule out that hydrogen bonding occurs between the tyrosine and either the backbone or side chains of other local residues. Such an event would flatten the tyrosine against the sidewall of the cavity rather than pointing into the channel and possibly causing an obstruction.

One outstanding question that is answered in this study is how P5C/GSA accesses the P5CDH active site. Previously it was unknown whether P5C/GSA exclusively used the channel to enter the second catalytic site or whether it could enter directly from bulk solvent. Experiments using D779Y and D779W with both exogenously added and endogenously produced P5C showed decreased P5CDH activity. In channeling assays using proline as a substrate, both D779Y and D779W showed very little NADH formation. Likewise, using exogenous L-P5C to determine kinetic parameters for the P5CDH domain revealed significantly lower kcat/Km values, predominantly due to the large decreases in kcat. If P5C/GSA were able to enter the P5CDH active site from a location other than the channel, the kinetic results would not have deviated from wild- type, especially when using exogenous P5C. It should be noted that both D779 mutants bind NAD+ similarly to wild-type and crystal structures show no global disruptions in protein folding (Dr. John Tanner, personal communication). Thus, the mutations did not structurally change or perturb a P5C entry/exit point that may have been near the P5CDH domain. Ultimately these results indicate that P5C must access the P5CDH domain using the channel. It also suggests that if exogenous P5C is able to enter the cavity from a site other than through the PRODH domain, it must do so upstream of the D779 residue.

A second elusive question in the PutA field is where and how hydrolysis of P5C takes place. It has been proposed that the large internal cavity is large enough to house several P5C and water molecules and may provide an environment for hydrolysis [2].

Based on the alternative substrate work using D779Y and D779W, it is tempting to suggest that P5C-GSA equilibrium favors the cyclic P5C at the point of these mutations in the channel. While succinate semialdehyde is missing a carbon and an amino group, perhaps the channeling improvement is due more to the linearity of the substrate rather than the shorter length. The larger D779W mutant shows nearly a 25-fold improvement when comparing the wild-type: mutant kcat/Km ratios for GSA and succinate semialdehyde.

This may suggest the ringed structure is more sterically hindered than the linear substrate, which is able to navigate the obstruction more efficiently. Based on the structure, D779 is located within the first third of the channel, leaving plenty of room for a hydrolysis reaction to occur downstream.

Making mutations along substrate channels is not new; other studies have reported similar experiments to validate or expand understanding of substrate channeling. Recently, two mutations were made along a surface crevice connecting two active sites in the Arabidopsis bifunctional enzyme dethiobiotin synthetase (DTBS)-diaminopelargonic acid aminotransferase (DAPAT-AT)[4]. The crevice is thought to channel the intermediate from DAPA-AT to DTBS. Mutants S360Y and I793W were made to obstruct the external crevice, and progress curves were monitored. The results showed the mutations caused a lag time of 10-12 minutes, whereas wild-type experienced no such lag, suggesting channeling of the intermediate was disrupted. Channel obstructions (βC170F, βC170W) have also been made in tryptophan synthase, causing the rate of intermediate channeling to slow10-fold in the phenylalanine mutant and over 1000-fold in the tryptophan mutant [10]. Steady-state and transient kinetic results indicated the βC170W not only hindered passage of intermediate between active sites, but also affected crosstalk between subunits [10]. In both examples substrate channeling was disrupted by placing bulky mutations along the channel, which provides precedence and validation for the research reported here.

Finally, the structures presented here show a snapshot of a cavity connecting two active sites, but it is necessary to think about the channel as being dynamic. It is well known that PutA undergoes a conformational change upon flavin reduction, and it has been proposed that a conserved ion pair (R456-E197) acts as a gate between the PRODH domain and the main cavity.[3] These two examples illustrate how the overall enzyme fluctuates, and it is reasonable that the topography of the channel changes as well. Future structural studies of the enzyme at different points of catalysis may show how the channel changes and could help further describe how P5C moves between active sites.

CONCLUSION

D779Y and D779W also exhibited lower P5CDH activity, suggesting that exogenous P5C must enter the channel upstream of D779. Replacing D779 with a smaller residue (D779A) had no effect on the catalytic and channeling properties of BjPutA showing that the carboxylate group of D779 is not essential for channeling. An identical mutation at D778 (D778Y) did not impact BjPutA channeling activity. Thus, D779 is optimally orientated so that replacement with the larger side chains of Tyr/Trp blocks P5C movment through the channel. The kinetic data reveal not only that bulky mutations at residue D779 hinder passage of P5C to the second active site, but also P5C must use the channel to efficiently access the P5CDH domain. Moreover, these mutants may be used to learn more about the hydrolysis event that is thought to take place within the channel.

Table. P5CDH Kinetic Parameters

	Glutamate-γ-Semialdehyde (GSA)			NAD[+b]
Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)	K_d (μM)
WT	0.42 ± 0.04	3.4 ± .12	8095 ± 822	0.60 ± 0.04
T348Y	0.42 ± 0.04	4.2 ± 0.15	10000 ± 1017	0.75 ± 0.06
S607Y	0.48 ± 0.03	4.5 ± 0.15	9375 ± 664	1.00 ± 0.04
D778Y	0.38 ± 0.02	3.8 ± 0.08	10000 ± 567	0.67 ± 0.04
D779A	0.38 ± 0.03	5.0 ± 0.14	13157 ± 1102	0.64 ± 0.05
D779Y	0.20 ± 0.03	0.02 ± 0.001	100 ± 15.8	0.65 ± 0.04
D779W	0.35 ± 0.15	0.003 ± 0.0005	8.6 ± 4.0	0.78 ± 0.05

[a] 0.01-6 mM P5C, 0.2 mM NAD^+, 600 mM NaCl, 0.25 μM enzyme, 50 mM potassium phosphate, pH 7.5

[b] 0.1-25 μM NAD^+, 0.25 μM enzyme, 50 mM potassium phosphate, pH 7.5

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