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Identification of Active Site Catalytic Residue in Isovaleryl-CoA Dehydrogenase from *Paracoccus denitrificans* Pd1222

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Abstract

The gene of *Pden_3633* in *Paracoccus denitrificans* Pd1222, Isovaleryl-CoA dehydrogenase gene (*IVDH*), was synthesized, cloned, expressed into *E. coli* BL21 (DE3) using pET24d vector, and purified as N-terminal Strep-Tagged enzyme (Karim and Hashim, 2016a; Karim and Hashim; 2016b). In current study, a Site-directed mutagenesis was used to identify the active site catalytic residue of this synthetic Sterp-Tag IVDH enzyme. Amino acid alignment showed that the E246 is the predicted active site catalytic residue. To substantiate the role of E246 as a catalytic residue, a mutant E246Q IVDH was constructed. Spectral properties of the mutant IVDH indicated that it was obtained as an apoprotein. Therefore, the protein was full reconstituted by incubation with flavin adenine dinucleotide (FAD) at a ratio 1: 20% (IVDH: FAD) molar excess. The results revealed that the reconstituted E246Q IVDH had no activity for isovaleryl-CoA. Furthermore, its UV/visible spectrum resulted from titration with isovaleryl-CoA did not induce quenching of the absorption at 364 and 440 nm regions or arise a new absorption at 598 nm as wild type did. Confirming that the mutant IVDH was unable to form charge transfer complex as a result of altering E246 and the later is the active site catalytic residue of *P. denitrificans* IVDH.

Introduction

Paracoccus denitrificans is a denitrifying, gram negative, cocci and non-motile bacterium. It contains all enzyme systems essentially to fully convert NO_3 - to N_2, It is responsible for emissions of the powerful green gas nitrous oxide (N_2O) from soils, All denitrification enzymes of *P. denitrificans* have been extensively studied making it a useful model for study of denitrification (Grönberg *et al*., 2004; Pitcher & Watmough, 2004; Field *et al*., 2008). In addition, the bacterium is more resemble to mitochondria than do other bacteria, it expressing for the respiratory transport chain very similar to that of the eukaryotic mitochondrion making it the favorite model for the study of oxidative phosphorylation (Yip *et al*., 2011; Hartop, 2014). *Paracoccus. denitrificans* Pd1222 genome has been sequenced in 2006 (Copeland *et al*., 2006). Two distinctive circular chromosomes (CP000489 and CP000490) and one plasmid (CP00049) have been detected. It has identified sixteen candidate Acyl-CoA dehydrogenase (ACAD) genes in *P. denitrificans* Pd1222 genome which are located among the two chromosomes and the plasmid. The expression of *ACAD* genes response to growth on different types of carboxylic acid as a sole carbon source is being investigated. Two of these *ACAD* genes (Pden_0948 and Pden_3633) have sequence are likely to be Isovaleryl CoA dehydrogenase gene (*IVDH*), the Pden_0948 located on chromosome 1 while the Pden_3633 (gene of interest) located on chromosome 2.

Materials and Methods

Site-directed mutagenesis or QuickChange method (Papworth *et al*., 1996) was used to identify the catalytic amino acid residue in recombinant IVDH. The technique requires PCR and using primers carrying

a mismatch responsible for the point mutation. The wild type Strep-Tag pET24d::IVDH plasmid, obtained by (Karim and Hashim, 2016a; Karim and Hashim; 2016b), was used as a template while synthetic forward and reverse complementary primers containing mismatch codon were used to introduce the point mutation in which glutamate at position 246 of the wild type Strep-Tag IVDH substituted with glutamine (E246Q).

Primers

Appropriate primers (table 1) were designed and synthesized by Eurofins (Genomics, UK.).

Table 1: Primers properties were used for QuickChange Mutagenesis

Name	Sequence	Tm [°C]	MW [g/mol]	GC%
IVDH E246Q For	'5 CTGGATTATCAACGCCTGGTG 3'	59.8	6437	52.4
IVDH E246Q Rev	'5 CACCAGGCGTTGATAATCCAG 3'	59.3	6415	52.4

Kits

Below, the kits used

· Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used for PCR reaction. The kit containing Phusion DNA Polymerase, 5X Phusion HF Buffer, 50 mM MgCl2 solution and DMSO.

· *Dpn* I (BioLabs, New England). The kit containing 1X CutSmart™ Buffer and *Dpn* I enzyme.

Procedure

The PCR reaction mixture and cycling condition are shown in tables (2) and (3) respectively. In the next step of the PCR, the PCR reaction product (table 4) was incubated with *Dpn* I at 37°C for 1 hour. After that, agarose gel electrophoresis was run (Fig. 1) and the mutated Strep-Tag pET24d::IVDH plasmid bands were extracted from agarose gel. The mutated construct was then transformed into *E. coli* BL21 (DE3) competent cells for expression and then purification (Karim and Hashim, 2016a).

Table 2: PCR reaction mixture

Component	Volume
Free nucleases H2O	31 μL
5X Phusion HF Buffer	10 μL
10 mM Deoxynucleotide (dNTP) Solution Mix	1 μL
Forward primer	(0.5 μM) 2.5 μL
Reverse primer	(0.5 μM) 2.5 μL
Template DNA	(50 ng) 1 μL
DMSO	1.5 μL
Phusion DNA Polymerase	0.5 μL
Total volume 50 μL	

Table 3: PCR cycling condition

Cycle step	Temp	Time	Cycle No.
Initial Denaturation	98°C	15 sec	1
Denaturation Annealing Extension	98°C 60°C 72°C	15 sec 30 sec 210 sec	30
Final extension	72°C	600 sec	1
Product hold at 4°C			

Table 4: Components of *Dnp* I reaction mixture

Component	Volume
Free nucleases H_2O	16 µL
PCR reaction product	10 µL
1X CutSmart™ Buffer	3 µL
Dpn I enzyme	1 µL
Total volume 30 µL	

Reconstitution of mutant enzyme

To reconstitute of mutant enzyme, the later was incubated with FAD according to Karim and Hashim (2016a).

Enzyme assay

IVDH activity calculated according to Engel (1981). The method depends on the reduction of 2,6-dichlorophenolindophenol (DCPIP) by an intermediate electron carrier, phenazine methosulfate (PMS), at the reaction temperature of 30°C. The final volume of 2500 µl of sodium phosphate buffer (100 mM, pH 7.6) included on, 37.5 µl DCPIP, 25 µl PMS, 12.5 - 25 µl of 15 – 60 µg IVDH and 25 µl isovaleryl-CoA (IV-CoA) was used as a reaction mixture. The assay method was started by adding different concentrations of IV-CoA (20 µM final Conc.).

Analysis of Spectral properties

Beckman DU640 UV/ Vis spectrophotometer was used to scan spectral properties of enzyme sample under aerobic conditions at 30 °C and by using 50 mM of sodium phosphate buffer, pH 8.0 as a dissolving solution.

Results and discussion

Amino acid alignment of the recombinant Strep-Tag P. d IVDH

DNA sequence alignment of the Strep-Tag P. d *IVDH* gene with deduced amino acids sequence was done by using ExPaSy translate software http://www.expasy.org/ and the results revealed a recombinant protein with 396 amino acids (fig. 1).

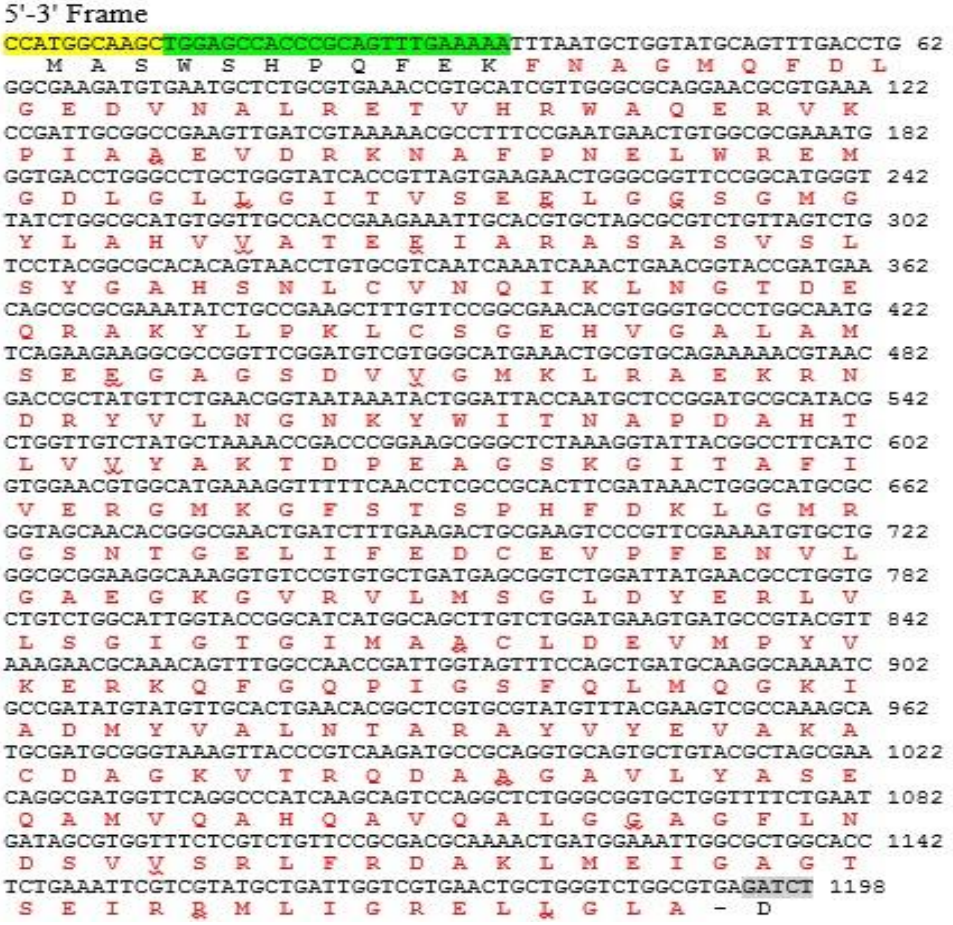

Figure 1: Nucleotide sequencing of the Strep-Tag P. d *IVDH* gene and deduced amino acids sequence. Yellow highlight: Nucleotide bases from *Nco* I in addition to caagc; Green highlight: Nucleotide bases encoded for the Strep-Tag; Gray highlight: Nucleotide bases from *Bgl* II which is un-translated.

Site directed mutagenesis

The members of ACADs share a common dehydrogenation mechanism in which the active site catalytic glutamate extracts a proton from an acyl-CoA substrate, but the position of the catalytic base is not conserved in the primary sequence. Glu376 of MCAD has been confirmed to be the catalytic residue from different sources and this base present within a highly conserved region among most other members of ACADs (Bross *et al*., 1990; Kim *et al*., 1993; Aoyama *et al*., 1995) with except IVDH and LCAD. Instead, Glu254 was confirmed to be the catalytic base in Human IVDH (Mohsen and Vockley, 1995; Tiffany *et al*., 1997; Mohsen *et al*., 2001) while Glu261 is the catalytic base in Human LCAD which is homologous to Glu254 of IVDH (Djordjevic *et al*., 1994).

By sequence comparison of conserved protein domains that concerned the active site catalytic residue, it seemed clear that the E246 is the presumed catalytic residue of the synthetic P. d IVDH. Therefore, a mutant E246Q P. d IVDH was constructed by using a site directed mutagenesis to substantiate the important of the E246 as a catalytic residue. The method introduced a point mutation in a double stranded

Strep-Tag *IVDH* gene by replacing guanine base at position 769 of the synthetic *IVDH* gene, position 5694 of the recombinant pET24d::IVDH, with cytosine to get CAA codon which encode for Q. Phusion DNA Polymerase, a novel *Pyrococcus*-like enzyme fused with a processivity-enhancing domain, was used for PCR reaction instead of *Thermus aquaticus* (Taq) DNA polymerase because the error rate of Phusion DNA Polymerase is approximately 50-fold lower than that of the Taq DNA polymerase and 6-fold lower than that of *Pyrococcus furiosus* DNA polymerase. Figure (2) shown the places where primers annealing with DNA template. The PCR product was treated with *Dpn* I restriction enzyme which digests methylated and hemimethylated DNA, Thus the parental plasmid is degraded whereas the mutated plasmid remains. The correctness of sequence mutant DNA was checked by nucleic acid sequencing (Eurofins Genomics GmbH, UK) using T7 promoter primer and the results showed 100% identify. The predicted band of the mutated plasmid was less than 4 Kb as it visualized in figure (3). The mutated plasmid was then transformed into *E. coli* BL21 (DE3) for expression and purification (fig. 4).

Figure 2: Annealing of primers with DNA template by site-directed mutagenesis. Yellow shadow: Sequences correspond to the original codons; Green shadow: Sequences of mismatch codons.

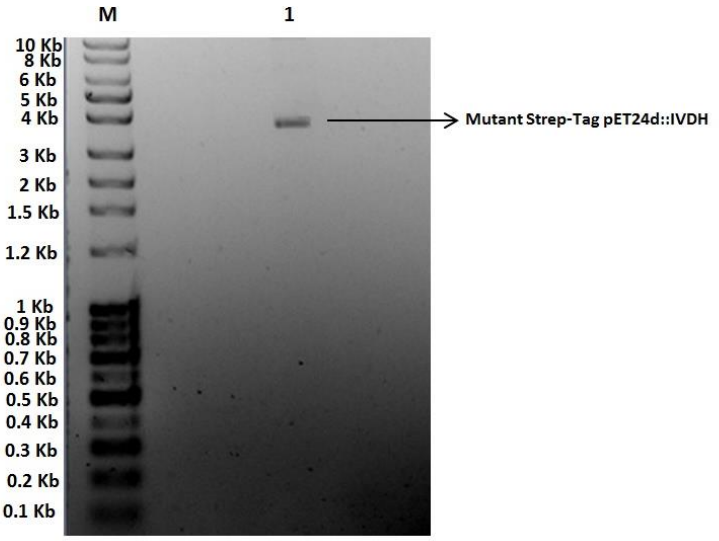

Figure 3: Agarose gel (1.2 %) of the mutated synthetic Strep-Tag pET24::IVDH. The gel was run for 1 hour at 100-120 volts. M: 2-Log DNA Ladder (0.1-10.0 Kb); Lane 1: Mutated synthetic Strep-Tag pET24d::IVDH

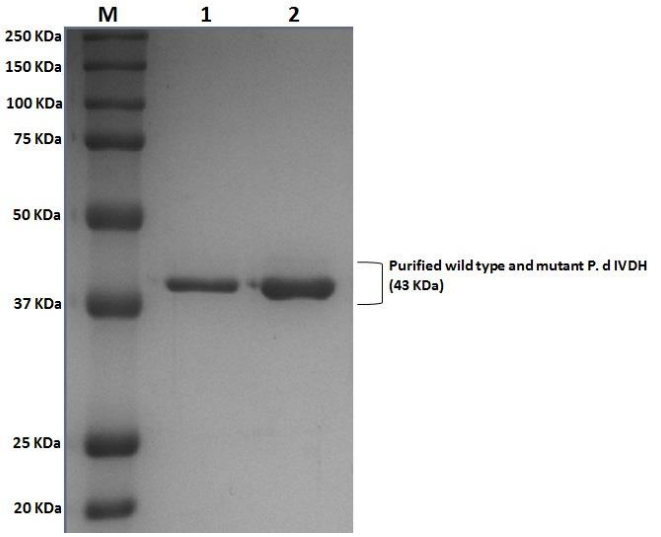

Figure 4: SDS gel electrophoretic pattern of the purified wild type P. d IVDH (lane 1) and its mutant E246Q (lane 2). M: Precision plus proteinTM standard marker. The SDS-PAGE was run on 12% and 4% resolving and stacking gels respectively using 110 Volt for 1-1.5 hrs, followed by Coomassie blue staining.

Reconstitution

To get on a holoprotein, the wild type and mutant recombinant IVDH have been reconstituted. And according to the experiment, the purified protein was re-purified after incubation duration (overnight) with the FAD to exclude the excess unbound FAD with wash. Thus, only the IVDH bound to the FAD (holoprotein) will eluted as a purified protein which then acquired an obvious yellow color resulted from FAD binding. Furthermore, the results showed that the molar excess ratio 1: 20% (IVDH: FAD) was the ratio which achieved a fully reconstituted of the protein (Fig. 5), one FAD per protein molecule which calculated by absorbance ratio 280/ 450 nm of approximately 5 (Banci *et al*., 2011).

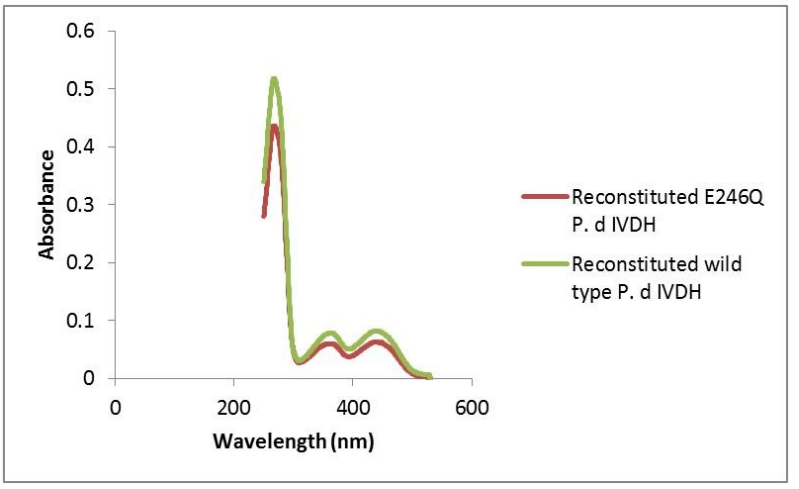

Figure 5: Spectral scan of reconstituted wild type (60 µM) and E246Q P. d IVDH (51 µM). The spectrum was recorded in 50 mM sodium phosphate buffer, pH 8.0, at 30 °C.

Titration of the reconstituted wild type P. d IVDH and its mutant

The formation of the charge-transfer complex, the charge transfer resulted between oxidized flavin and Isovaleryl-CoA and also between reduced flavin and 3-methylcrotonyl-CoA product, was studied by aerobic titration of the reconstituted wild type and mutant E246Q P. d IVDH with the Isovaleryl-CoA substrate and the absorbance changes was then monitored.

Beckman DU640 spectrophotometer was used to scan spectral properties. The results in figure (6) showed the most spectral changes resulted from adding increasing amounts of the Isovaleryl-CoA to The reconstituted wild type IVDH which including quenching of the absorption at 364 and 440 nm regions. In addition to a shoulder at 311 nm and a new absorbance band extending between 520 and 800 nm with absorption peak at 598 nm. As for the reconstituted mutant E246Q IVDH, the spectral results did not show a quenching at the corresponding regions 364 and 440 nm (fig. 7). Alternatively, 5 and 9 nm red shifts of the absorbance have been detected in the equivalent 364 and 440 nm regions to become 369 and 449 nm respectively. Furthermore, mutant IVDH does not have a peak of the characteristic absorbance band at 598 nm whereas the shoulder at 311 nm was very narrow. The quenching of absorbance at 440 nm, which can be attributed to the reduction of the enzyme bound FAD, with the concomitant appearance of the broad absorption band with peak at 598 nm provide evidence for the formation of the charge-transfer complex. Also, broad peak's values of shoulder at 311 nm contribute dramatically to the interaction between the enzyme-bound flavin and product (Auer and Frerman, 1980).

Many studies provide a proof for the formation of the charge transfer complex of the ACADs based on distinct spectral changes which include a characteristic quenching in the absorption at 450 nm, or slightly blue shift, with appearance of a new absorption band at ~580 nm. While many mutant forms of these enzymes showed red shift of the absorbance at the corresponding 450 nm and/ or loss of quenching at this region (Mohsen *et al*., 1998; Bharathi *et al*., 2013; Mohsen and Vockley, 2015). The results of this study was in similar with a study by Mohsen and Vockley (1995) whose confirmed that the E254 is a catalytic residue in human IVDH, they generated a catalytic base mutant of human IVDH (E254Q) and they found no catalytic activity was detected with the Isovaleryl-CoA. Also, the authors showed that the titration of the mutant enzyme with the substrate does not gives quenching at the corresponding region 450 nm. Instead, it was red shifted. Likewise, the absorption band at ~580 nm was blunted Indicate that the mutant enzyme was unable to form the charge transfer complex. Based on these findings that consistent with the results of current study, it could confirm that the E246 is a catalytic residue of P. d IVDH.

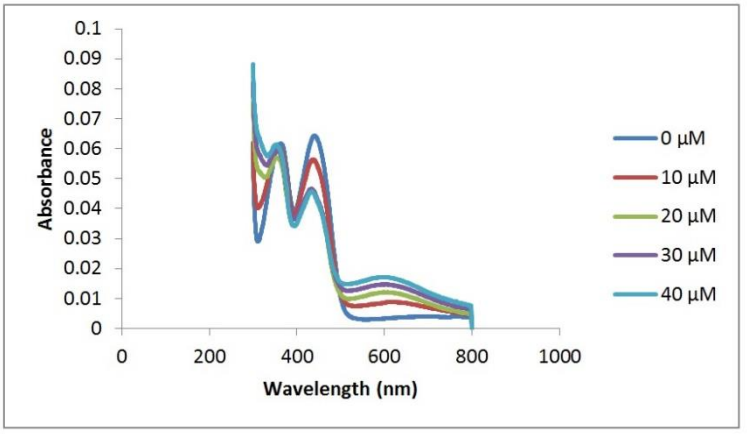

Figure 6: Absorption spectrum of reconstituted wild type P. d IVDH titrated with increasing amounts of Isovaleryl-CoA. The titration conducted aerobically in 50 mM sodium phosphate buffer, pH 8.0, at 30 °C.

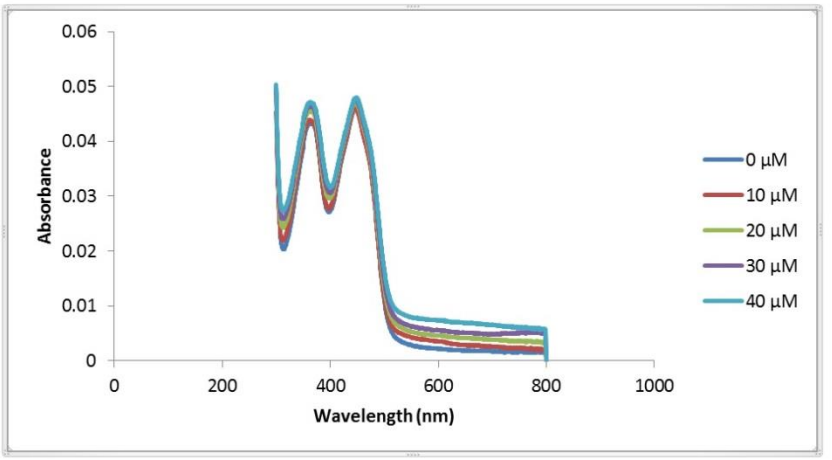

Figure 7: Absorption spectrum of reconstituted mutant (E246Q) P. d IVDH titrated with increasing amounts of Isovaleryl-CoA. The titration conducted aerobically in 50 mM sodium phosphate buffer, pH 8.0, at 30 °C.

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