



IDENTIFICATION OF ISOVALERYL-COA DEHYDROGENASE CATALYTIC RESIDUE IN *PARACOCCLUS DENITRIFICANS* PD1222

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Abstract

In previous studies, the gene of *Pden_3633* [Isovaleryl-CoA dehydrogenase gene (*IVDH*)] in *Paracoccus denitrificans* Pd1222, was synthesized, cloned, expressed into *E. coli* BL21 (DE3) using pET24d vector and purified as N-terminal Strep-Tagged enzyme. In current study, a Site-directed mutagenesis used to identify the active site catalytic residue of this synthetic Strep-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.

Key words: catalytic residue, Isovaleryl-CoA dehydrogenase, *Paracoccus denitrificans*, Reconstitution.

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.*, 2000; Pitcher and Watmough, 2000; 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.

Methods and Materials

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 (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

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Table 1: Primers properties used for QuickChange Mutagenesis.

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

synthesized by Eurofins (Genomics, UK).

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

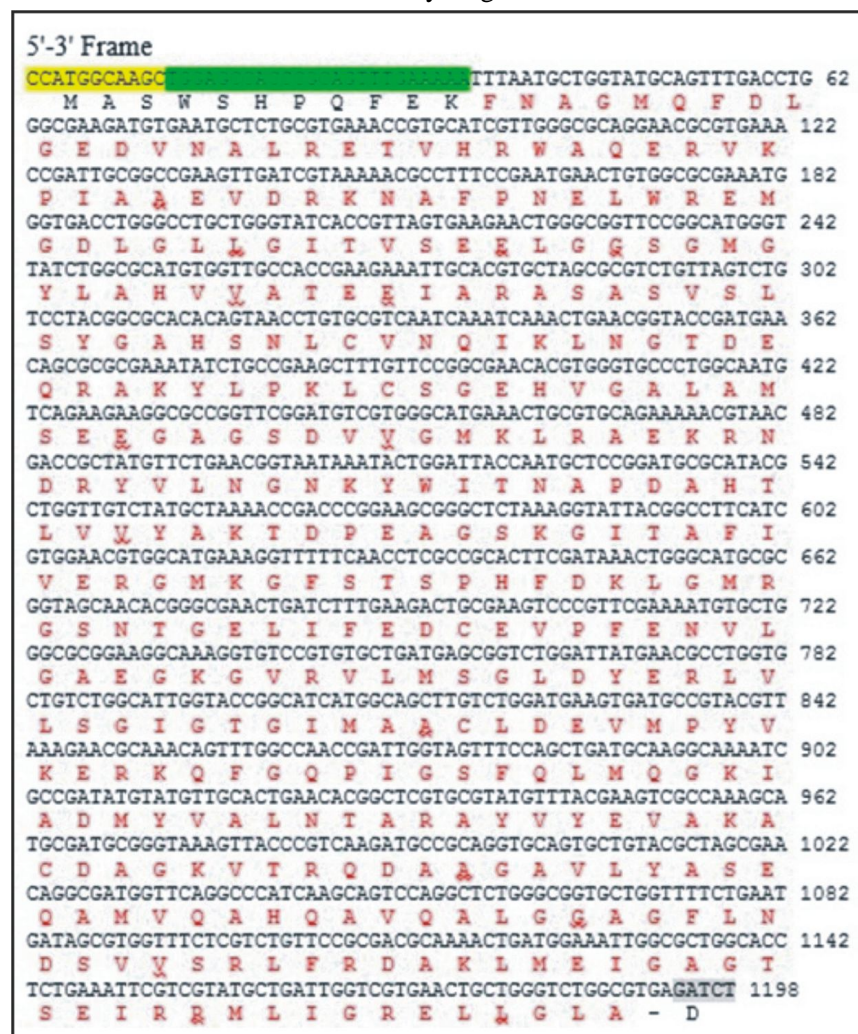


Fig. 1: Nucleotide sequencing of the Strep-Tag P. d IVDH gene and deduced amino acids sequence. Yellow highlight: Nucleotide bases from *Nco* I in addition to caagc; Green highlight: Nucleotide bases encoded for the Strep-Tag; Gray highlight: Nucleotide bases from *Bgl* II which is un-translated.

shown in (Table 2) and (Table 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 and the mutated Strep-Tag pH 8.0 as a dissolving solution.

Results and Discussions

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).

Site directed mutagenesis

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).

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 (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,

Table 2: PCR reaction mixture.

Component	Volume
Free nucleases H ₂ O	31 μ L
5X Phusion HF Buffer	10 μ L
10 mM Deoxynucleotide (dNTP) Solution Mix	1 μ L
Forward primer	(0.5 μ M) 2.5 μ L
Reverse primer	(0.5 μ M) 2.5 μ L
Template DNA	(50 ng) 1 μ L
DMSO	1.5 μ L
Phusion DNA Polymerase	0.5 μ L
Total volume 50 μ L	

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

Table 3: PCR cycling condition.

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

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.*, 1997). 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



Fig. 2: Annealing of primers with DNA template by site-directed mutagenesis (A) and Agarose gel (1.2 %) of the mutated synthetic Strep-Tag pET24d::IVDH (B). Yellow shadow: Sequences correspond to the original codons; Green shadow: Sequences of mismatch codons (A). Agarose 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 (B).

Table 4: Components of *Dpn* I reaction mixture.

Component	Volume
Free nucleases H ₂ O	16 μ L
PCR reaction product	10 μ L
1X CutSmart™ Buffer	3 μ L
<i>Dpn</i> I enzyme	1 μ L
Total volume 30 μ L	

recombinant pET24d::IVDH, with cytosine to get CAA codon which encode for Q. The places where primers annealing with DNA template shown in (Fig. 2A). 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 (Fig. 2B). The mutated plasmid was then transformed into *E. coli* BL21 (DE3) for expression and purification (Fig. 3).

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,

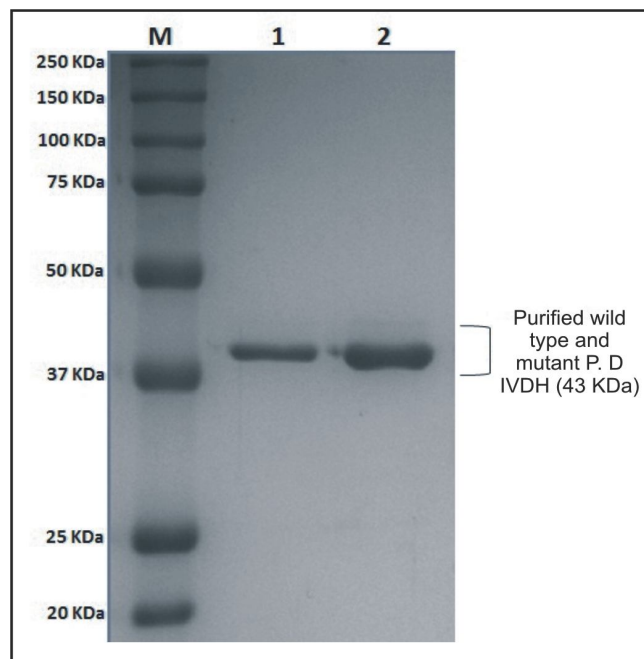


Fig. 3: SDS gel electrophoretic pattern of the purified wild type P. d IVDH (lane 1) and its mutant E246Q (lane 2). M: Precision plus protein TM standard marker. The SDS-PAGE was run on 12% and 4% resolving and stacking gels respectively using 110 Volt for 1-1.5 hrs, followed by Coomassie blue staining.

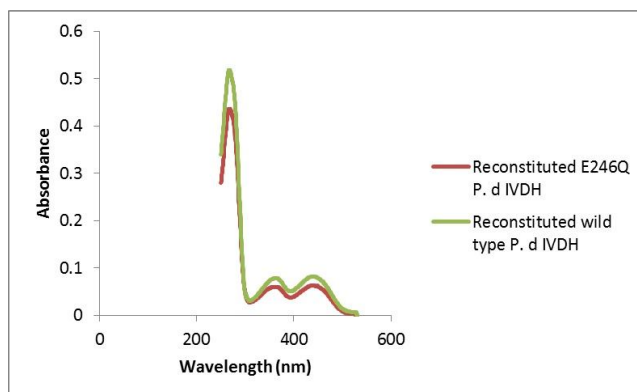


Fig. 4: Spectral scan of reconstituted wild type (60 μ M) and E246Q P. d IVDH (51 μ M). The spectrum was recorded in 50 mM sodium phosphate buffer, pH 8.0, at 30°C.

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. 4), one FAD per protein molecule which calculated by absorbance ratio 280/ 450 nm of approximately 5 (Banci *et al.*, 2011).

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

The formation of the charge-transfer complex, the

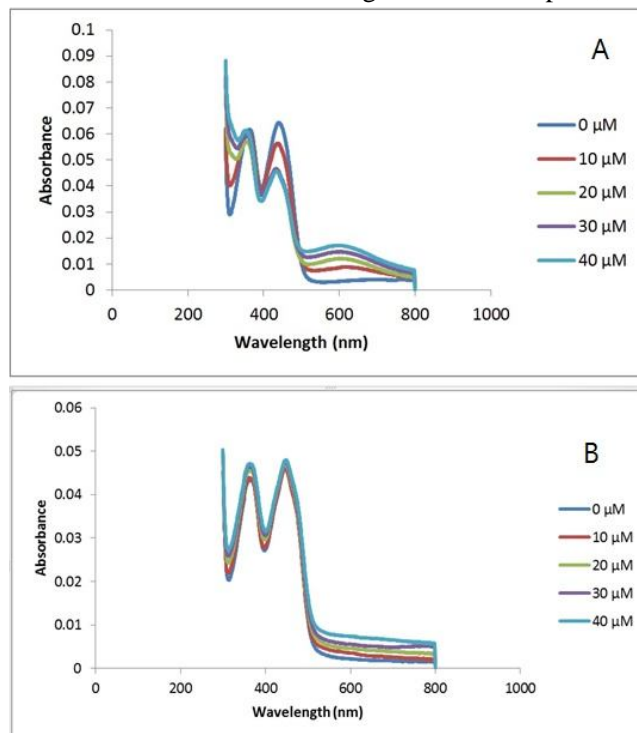


Fig. 5: Absorption spectrum of reconstituted wild type P. d IVDH (A) and reconstituted mutant (E246Q) P. d IVDH (B) titrated with increasing amounts of Isovaleryl-CoA. The titration conducted aerobically in 50 mM sodium phosphate buffer, pH 8.0, at 30°C.

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 (Fig. 5A) 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. 5B). 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.

Conclusion

Results of the current study showed that the titration of the mutant E246Q IVDH enzyme with the Isovaleryl-CoA substrate does not gives quenching at the 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 we can concluded that the E246 is a catalytic residue of P. d IVDH.

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