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Role of glutamine 148 of human 15-hydroxyprostaglandin dehydrogenase in catalytic oxidation of prostaglandin E_2

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Abstract—NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a member of the short-chain dehydrogenase/reductase (SDR) family, catalyzes the first step in the catabolic pathways of prostaglandins and lipoxins. This enzyme oxidizes the C-15 hydroxyl group of prostaglandins and lipoxins to produce 15-keto metabolites which exhibit greatly reduced biological activities. A three-dimensional (3D) structure of 15-PGDH based on the crystal structures of the levodione reductase and tropinone reductase-II was generated and used for docking study with NAD^+ coenzyme and PGE_2 substrate. Three well-conserved residues among SDR family which correspond to Ser-138, Tyr-151, and Lys-155 of 15-PGDH have been shown to participate in the catalytic reaction. Based on the molecular interactions observed from 3D structure of 15-PGDH, we further propose that Gln-148 in 15-PGDH is important in properly positioning the 15-hydroxyl group of PGE_2 by hydrogen bonding with the side-chain oxygen atom of Gln-148. This residue is found to be less conserved and replaceable by glutamyl, histidinyl, and asparaginyl residues in SDR family. Accordingly, site-directed mutagenesis of Gln-148 of 15-PGDH to alanine, glutamic acid, histidine, and asparagine (Q148A, Q148E, Q148H, and Q148N) was carried out. The activity of mutant Q148A was not detectable, whereas those of mutants Q148E, Q148H, and Q148N were comparable to or higher than the wild type. This indicates that the side-chain oxygen or nitrogen atom at position 148 of 15-PGDH plays an important role in anchoring C-15 hydroxyl group of PGE_2 through hydrogen bonding for catalytic reaction.

1. Introduction

Prostaglandins and lipoxins are two families of biologically potent fatty acids derived from arachidonic acid through cyclooxygenase and lipoxygenase pathways, respectively. Prostaglandins and lipoxins are rapidly metabolized by the initial oxidation of the 15(*S*)-hydroxyl group catalyzed by 15-hydroxyprostaglandin dehydrogenase (15-PGDH).[1] Two different types of 15-PGDH have been recognized. Type I is NAD^+ specific, while Type II is $NADP^+$ preferred. Type I is more prostaglandin and lipoxin specific and exhibits a low K_m for prostaglandins and lipoxins, whereas Type II has a much broader substrate specificity and shows a high K_m for prostaglandins.[2] In fact, Type II was later found to be identical with carbonyl reductase.[3] Therefore, Type I has been considered the key enzyme responsible for the biological inactivation of prostaglandins and lipoxins. Studies on prostaglandin and lipoxin catabolism have so far been focused on the Type I enzyme.

Type I 15-PGDH (hereafter referred to as 15-PGDH) is active as a homodimer with a subunit M_w of 29 kDa.[4] Sequence alignments indicate that it belongs to the short-chain dehydrogenase/reductase (SDR) family. A consensus sequence of Tyr-X-X-X-Lys as well as a nearby generally conserved upstream serine residue in the middle part of the enzymes characterizes this protein family.[5] Furthermore, a glycine-rich motif Gly-X-X-X-Gly-X-Gly typical of a Rossmann

Abbreviations: 15-PGDH, NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase; SDR, short-chain dehydrogenase/reductase; DTT, dithiothreitol; PVDF, polyvinylidene; GST, glutathione *S*-transferase; SCR, structural conserved regions; VR, variable regions.

Keywords: Prostaglandins; 15-Hydroxyprostaglandin dehydrogenase; Molecular modeling; Site-directed mutagenesis.

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fold is found near the N-terminus and is thought to be part of the putative coenzyme binding site.[6] Three well-conserved residues among SDR family (the triad Ser-138, Tyr-151, and Lys-155 in 15-PGDH) are known to be involved in the catalytic reaction, based on mutagenesis[7–13] and X-ray analysis.[14–21] We also have identified Tyr-151, Lys-155, and Ser-138 in 15-PGDH as being a catalytically important triad by site-directed mutagenesis.[22,23] Tyr-151 is the most conserved residue in SDR and probably acts as a general base in the catalytic reaction. Lys-155 has a dual role: fixation of the nicotinamide ring of the coenzyme and lowering the pK_a value of the phenolic group of Tyr-151. Finally, Ser-138 is also conserved in the SDR family and assists the proper positioning of the hydroxyl or carbonyl group of the substrate. We have also identified Thr-11, Ile-74, Asn-91, Cys-182, Val-186, and Thr-188 in 15-PGDH as being involved in interacting with NAD^+ by site-directed mutagenesis.[24–27]

Enzymes in the SDR family are either NAD^+ or $NADP^+$ specific or utilize both coenzymes. Either nucleotide stereospecifically transfers a hydride from or to the carbon atom of the substrates in position 4 of the nicotinamide ring. Although the three-dimensional structure of 15-PGDH has yet to be determined, crystal structures of the binary and tertiary complexes of other members of the SDR family, such as levodione reductase from *Corynebacterium aquaticum*[28] and tropinone reductase-II from *Datura stramonium*,[29] have been elucidated. A 3D structure of 15-PGDH based on the crystal structures of the levodione reductase and tropinone reductase-II was generated and used for docking study with the NAD^+ coenzyme. A ternary structure-based sequence alignment with 10 members of the SDR family of known three-dimensional structures has suggested a model which relates the observed low degree of sequence identity to quite similar folding patterns and nearly identical distributions of residues involved in catalysis. The reliability of this model is supported by our publications of studies on 15-PGDH active sites using site-directed mutagenesis. From the 3D structural analysis of 15-PGDH-NAD^+-PGE_2 complex and the sequence analysis of the SDR family, we propose that Gln-148 participates in the catalytic oxidation of PGE_2 in 15-PGDH. Site-directed mutagenesis study of this residue supports this contention as revealed in the current report.

2. Results and discussion

15-PGDH is a member of the SDR family of about 60 enzymes.[5] Even though the overall homology among these enzymes is low, there are a number of amino acid residues which are highly conserved. Conservation of these residues would suggest that they serve a common role in all of the proteins in which they are present. The roles of these residues might include binding of the nucleotide cofactor, conservation of an important structural feature of the proteins or involvement in the catalytic mechanism. Several conserved residues in every single SDR enzyme presently known such as residues corresponding to Ser-138,[8] Tyr-151,[7] Lys-155,[7] Cys-182,[12] and Thr-188[25] of human placental 15-PGDH were found to be essential for catalytic activity. Recently, we also found that Gln-15, Asp-36, and Trp-37 of human placental 15-PGDH appeared to be involved in coenzyme specificity.[30]

Although the crystal structure of 15-PGDH is yet to be determined, the crystal structures of the binary and tertiary complexes of other members of the SDR family, such as levodione reductase from *C. aquaticum* and tropinone reductase-II from *D. stramonium* have been elucidated. A 3D structure of 15-PGDH based on the crystal structures of the levodione reductase and tropinone reductase-II was generated and used for docking study with NAD^+ coenzyme and PGE_2 substrate. Sequence alignment of levodione reductase, tropinone reductase-II, and 15-PGDH as well as other SDR enzymes is shown in Figure 1. Ser-152, Gln-162, Tyr-165, and Lys-169 of levodione reductase and Ser-146, Gln-156, Tyr-159, and Lys-163 of tropinone reductase-II which correspond to Ser-138, Gln-148, Tyr-151, and Lys-155 of 15-PGDH were shown to make interactions with cofactor and substrate. By examining the high-resolution crystal structures of the apo form, binary and ternary complexes, and biochemical data gathered for many SDR family members, we have proposed a reaction mechanism for 15-PGDH.[7–21] At present, the 'Ser-Tyr-Lys catalytic triad' is considered to be important in SDR catalysis, whereby the side-chain oxygen of the tyrosine residue functions as an acid–base catalyst for proton transfer. These conserved residues are in fact located around the hydroxyl group of PGE_2 in the ternary complex of 15-PGDH, and the side-chain oxygen of Tyr-151 residue points toward the face of the cofactor nicotinamide ring. We have identified Ser-138, Tyr-151, and Lys-155 in 15-PGDH as being a catalytically important triad by site-directed mutagenesis[7,8] and homology modeling.[31] By homology modeling, docking, molecular dynamics simulation, and site-directed mutagenesis of 15-PGDH, we also can propose that the Gln-148 in 15-PGDH is important in the proper positioning of the C-15 hydroxyl group of PGE_2 substrate during catalytic reaction.

A proposed 3D model of a tertiary complex of 15-PGDH-NAD^+-PGE_2 is shown in Figure 2. The model shows that the 15-hydroxyl group of the PGE_2 is anchored by hydrogen bonding with the hydroxyl group of Ser-138 and the side-chain oxygen atom of Gln-148. Among the SDR enzymes listed in Figure 1, residue corresponding to Ser-138 is basically conserved, whereas that corresponding to Gln-148 can be glutamine as well as glutamic acid, histidine, and asparagine. Based on this information, site-directed mutagenesis of Gln-148 of 15-PGDH to alanine, glutamic acid, histidine, and asparagine (Q148A, Q148E, Q148H, and Q148N) was carried out (Table 1). The activity of mutant Q148A was not detectable, whereas those of mutants Q148E, Q148H, and Q148N were comparable to or higher than

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                       138                148 151   155
                        ▼                  ▼   ▼     ▼
15-PGDH    130 G G I I I N M S S L A G L M - P V A Q Q P V Y C A S K H G I V G F T R 163
LevoR      144 S G M V V N T A S V G G I R - G I G N Q S G Y A A A K H G V V G L T T 177
TR-II      138 R G N V V F I S S V S G A L - A V P Y E A V Y G A T K G A M D Q L T R 171
17β-HSD-1  134 S G R V L V T G S V G G L M - G L P F N D V Y C A S K F A L E G L C E 167
NPG        132 W G R I I N I S S V N G V K - G Q A G Q T N Y S A A K A G V I G F T K 165
3-O(K)AR   128 F G R I I N I G S V V G S T - G N P G Q T N Y C A A K A G V V G F S K 161
FabG       128 F G R I I T I G S V V G S M - G N P G Q T N Y C A A K A G L I G F S K 161
AGR-L      132 T G D I I V T C S I A G H F - P T Y W E P V Y S G S K W A I T S F V Q 165
POxidoR    124 R G H V V N I S S I A G A Y - N F G G N S T Y H A T K A A I H M L S R 157
LOxidoR    131 N G L V I N V S S G L S N L - A D G A H P I Y N L T K A G V H F Y S D 164
OxidoR     132 F G R I V N V A S L A G A V P G A A G H T L Y A A T K G F L V K F S Q 166
LDR        144 S G M V V N T A S V G G I R - G I G N Q S G Y A A A K H G V V G L T R 177
ACoR       135 E G R I I S I S S I I G Q A - G G F G Q T N Y S A A K A G M L G F T K 168
3-KCoAR    135 G G R V I N I S S I I G Q A - G G F G Q T N Y S A A K A G M L G F T K 168
Glu-1-D    137 G G S I V N I S S V S G F R - P Q P N N I A Y V A A K H G V V G M T K 170
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Figure 1. Comparison of sequences of the SDR family. Following enzyme sequences are co-listed with that of human 15-PGDH in the figure. Levodione reductase from *Corynebacterium aquaticum* (LevoR); tropinone reductase-II from *Datura stramonium* (TR-II); human 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD-1); *Azospirillum brasilense* nodulation protein G (NPG); *Haemophilus influenzae* 3-oxoacyl-[acyl-carrier-protein] reductase (3-ketoacyl-acyl carrier protein reductase) [3-O(K)AR]; *Pasteurella multocida* FabG (FabG); *Agrobacterium tumefaciens* str. AGR_L_1076p (AGR-L); *Ralstonia solanacearum* Probable oxidoreductase (POxidoR); *Lactococcus lactis* subsp. lactis oxidoreductase (LOxidoR); *Caulobacter crescentus* oxidoreductase (OxidoR); *Leifsonia aquatica* levodione reductase ((6R)-2,2,6-trimethyl-1,4-cyclohexanedione reductase) (LDR); *Bacillus cereus* acetoacetyl-CoA reductase (ACoR); *Bacillus megaterium* 3-ketoacyl-CoA reductase PhaB (3-KCoAR); *Oceanobacillus iheyensis* glucose 1-dehydrogenase (Glu-1-D).

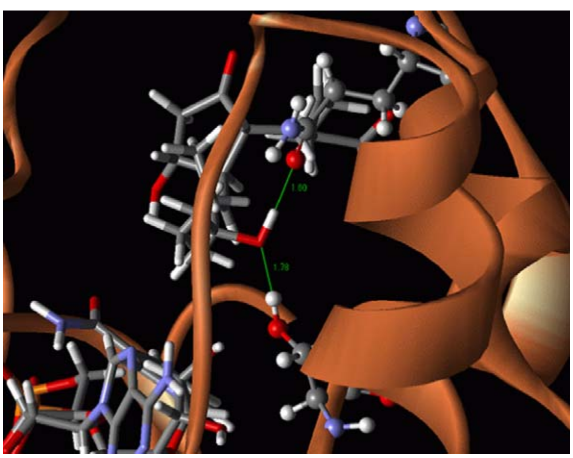

Figure 2. Constructed 3D structural model of 15-PGDH-NAD$^+$-PGE$_2$ complex. The model was generated by homology molecular modeling based on the crystallographic structures of levodione reductase and tropinone reductase-II. The model indicates the interactions of hydroxyl group of PGE$_2$ with residues Ser-138 and Gln-148 of the wild type 15-PGDH.

Table 1. 15-PGDH activity in *E. coli* expressing GST fusion enzymes of wild type and its mutants

Transformant	Activity NADH (μM/min/μg protein)
pGEX-2T-GST-PGDH	2.8
pGEX-2T-GST-PGDH Q148A	ND
pGEX-2T-GST-PGDH Q148E	7.5
pGEX-2T-GST-PGDH Q148H	16.0
pGEX-2T-GST-PGDH Q148N	17.8

The enzyme was assayed fluorometrically as described in Section 3. The concentration of PGE$_2$ and NAD$^+$ used was 17.4–99.4 μM and 60–4000 μM, respectively. 15-PGDH and its mutants were expressed as a GST fusion enzyme using pGEX-2T vector as described in Section 3. pGEX-2T-GST-PGDH: wild type 15-PGDH. Notation of mutants is indicated in a similar manner except adding the site of mutation and the amino acid being mutated to. ND: No detectable activity.

the wild type. This indicates that the side-chain oxygen or nitrogen atom at position 148 plays an important role in anchoring 15-hydroxyl group of PGE$_2$ through the hydrogen bonding. Figure 2 indicates that the Ser-138 and Gln-148 interact with the 15-hydroxyl group of PGE$_2$ substrate. As seen in Figure 3, molecular dynamics (MD) simulations on mutants Q148A, Q148E, Q148H, and Q148N were carried out to study the interactions of PGE$_2$ with the side-chains of mutants. The results showed that the side-chain of the mutant Q148A (Fig. 3A) cannot form hydrogen bonding with the 15-hydroxyl group of PGE$_2$ resulting in a wobbling motion of the hydroxyl group. However, Figures 3(B, C, and D) indicates that the C-15 hydroxyl group of PGE$_2$ is stabilized by the side-chain oxygen atom of mutants Q148E and Q148N or by the side-chain nitrogen atom of the mutant Q148H.

Mutants Q148E, Q148H, and Q148N had higher activity than the wild type. Especially, the activities of mutants Q148H and Q148N were increased nearly 6-fold (Table 2). However, k_{cat}/K_m ratios of mutants Q148H and Q148E were decreased 3-fold for PGE$_2$ and 3- and 41-fold for NAD$^+$, respectively, because of significant increase in K_m for NAD$^+$ with both mutants. These results indicate that Gln-148 of the 15-PGDH is involved in the interaction with both PGE$_2$ and NAD$^+$ and contributes to the full catalytic activity of 15-PGDH.

In summary, the side-chain oxygen or nitrogen atom of the amino acid at position 148 may play an important role in catalytic oxidation of PGE$_2$ as glutamic acid, histidine, and asparagine mutants exhibited higher activity and alanine mutant showed no activity at all. C-15 hydroxyl group is placed in between oxygen atoms of the side-chains of Ser-138 and Gln-148. A proton can then be easily transferred from the alcohol group of

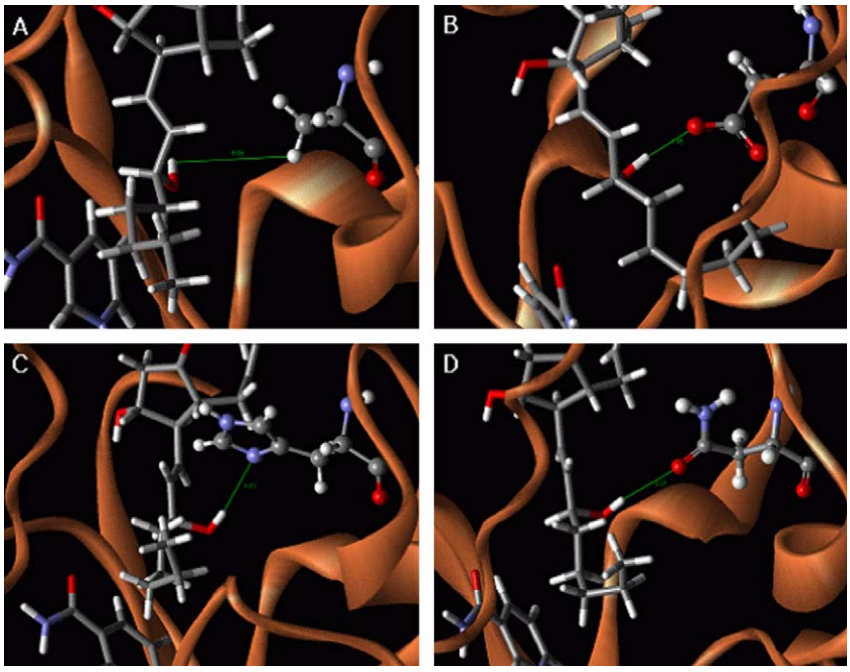

Figure 3. Constructed 3D structural model of 15-PGDH-NAD^+-PGE_2 complex. The model was generated by homology molecular modeling based on the crystallographic structures of levodione reductase and tropinone reductase-II. The model indicates the interactions of hydroxyl group of PGE_2 with residue Ala-148 of the mutant Q148A (A), the residue Glu-148 of the mutant Q148E (B), the residue His-148 of the mutant Q148H (C), and the residue Asn-148 of the mutant Q148N (D).

Table 2. K_m and k_{cat}/K_m values for 15-PGDH mutants

	PGE_2			NAD^+		
	K_m (μM)	$k_{cat}(s^{-1})$	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Wild type	3.4 ± 0	50 ± 6	1.5×10^7	38 ± 4	47 ± 4	1.24×10^6
Q148H	58.4 ± 8	329 ± 57	0.6×10^7	342 ± 39	266 ± 26	0.78×10^6
Q148N	24.5 ± 3	297 ± 32	1.2×10^7	240 ± 20	299 ± 51	1.25×10^6
Q148E	25.8 ± 4	125 ± 26	0.5×10^7	3273 ± 331	112 ± 30	0.03×10^6

The K_m value of the purified enzymes was determined by Lineweaver-Burk plots. Data processing and curve fitting were assisted by using GRAPHPAD software. The K_m value for PGE_2 was determined using NAD^+ (60–4000 μM) as a coenzyme. The K_m value for NAD^+ was determined using PGE_2 (17.4–99.4 μM) as a substrate. The enzyme activity was assayed fluorometrically as described in Section 3.

PGE_2 to the unprotonated form of residue Tyr-151. This process facilitates a hydride transfer from the C-15 position of the PGE_2 to the C-4 position of the nicotinamide ring of NAD^+ (Fig. 4). Ser-138 and Gln-148 either orients the PGE_2 properly or stabilizes the transition intermediate during the oxidation process or both.

3. Experimental

3.1. Materials

NAD^+, NADH, glutathione (GSH)-Sepharose 4B, sodium dodecylsulfate (SDS) and dithiothreitol (DTT) were obtained from Sigma. PGE_2 was from Cayman Chemicals. Cloned *pfu* DNA polymerase was from Stratagene. *Dpn*I endonuclease was from New England BioLabs. Protein A-HRP was from Transduction Laboratories. Polyvinylidene fluoride (PVDF) membrane was obtained from the Millipore Corp. GST gene fusion pGEX-2T expression vector was from Pharmacia Corp. The QIAprep Spin Plasmid Miniprep Kit was from QIAGEN. ECL^+ Plus Western blotting detection system RPN 2132 was from Amersham Life Science. Oligonucleotide primers were synthesized by the Macromolecular Structure and Analysis Facility of the University of Kentucky.

3.2. Molecular modeling of the 15-PGDH

The amino acid sequences of the human 15-hydroxyprostaglandin dehydrogenase (15-PGDH) [AC: 31542939] were derived from cDNA sequence as described previously.[4] Based on the crystal structure of the levodione reductase from *C. aquaticum*[28] and Tropinone reductase-II from *D. stramonium*,[29] homology-based enzyme model was built using the program Insight II (Accelrys, San Diego, CA). In order to find structurally conserved regions, sequences of the reference proteins were manually aligned based on its secondary structure. The sequence of the corresponding human 15-PGDH protein was then aligned to the reference proteins and the

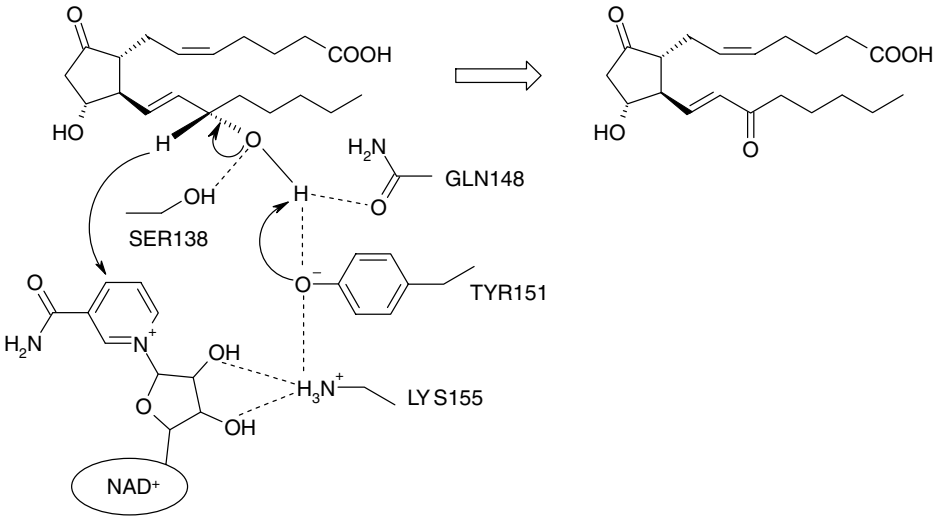

Figure 4. Proposed catalytic mechanism of 15-PGDH.

coordinates were assigned within the structural conserved regions (SCR) as we did in modeling the wild type enzyme.[31] Loops or variable regions (VR), which are located between SCRs, were found exclusively at the surface of the protein, not interacting with the active site. Coordinates for the VRs were either directly generated or assigned from known crystal structures. The conformations of the side chains were retained in conserved positions and the statistically most likely rotamer (rotational position of side-chain based on analysis of all known protein structures containing that amino acid) was chosen when no conformational information was available. The final structure was refined by energy minimization using the Discover/InsightII with Amber force field.[32] The multiple sequence alignment was performed using CLUSTALW[33] implemented in the BIOEDIT program.[34] All color figures shown are generated using Weblab viewer (Accelrys, San Diego, CA).

3.3. Site-directed mutagenesis

All site-directed mutagenesis of human placental 15-PGDH cDNA was performed by QuikChange site-directed mutagenesis method.[35] Four pairs of PCR primers were used to perform PCR. The oligonucleotides used for mutagenesis had the following sequences: for mutagenesis of glutamine 148 to alanine 148 (Q148A), 5′ GGA CTC ATG CCC GTT GCA CAG GCT CCG GTT TAT TGT GCT TCA AAG 3′ and 5′ CTT TGA AGC ACA ATA AAC CGG AGC CTG TGC AAC GGG CAT GAG TCC 3′; glutamine 148 to asparagine 148 (Q148N), 5′ GGA CTC ATG CCC GTT GCA CAG AAA CCG GTT TAT TGT GCT TCA AAG 3′ and 5′ CTT TGA AGC ACA ATA AAC CGG TTT CTG TGC AAC GGG CAT GAG TCC 3′; glutamine 148 to histidine 148 (Q148H), 5′ GGA CTC ATG CCC GTT GCA CAG CAC CCG GTT TAT TGT GCT TCA AAG 3′ and 5′ CTT TGA AGC ACA ATA AAC CGG GTG CTG TGC AAC GGG CAT GAG TCC 3′; glutamine 148 to glutamic acid 148 (Q148E), 5′ GGA CTC ATG CCC GTT GCA CAG GAA CCG GTT TAT TGT GCT TCA AAG 3′ and 5′ CTT TGA AGC ACA ATA AAC CGG TTC CTG TGC AAC GGG CAT GAG TCC 3′, where the underlined bases indicate the bases that were changed. *Pfu* DNA polymerase was used for PCR. The PCR product was treated with *Dpn*I endonuclease to digest the parental DNA template. The DNA sequences of the mutants were confirmed by DNA sequencing.

3.4. Expression and purification of 15-PGDH

Expression and purification of 15-PGDH were carried out as described previously.[26,27] Briefly, the altered cDNA prepared above was inserted between *Bam*HI and *Eco*RI sites of the pGEX-2T expression vector.[26] The recombinant plasmid was used to transform *E. coli* BL-21 LysS. Cells were grown in 500 mL YT medium containing 50 μg/mL ampicillin at 37 °C with shaking (250 rpm) until OD_{600} reached 0.6. IPTG was added to a final concentration of 1 mM and cells were allowed to grow for additional 12 h at 25 °C. Cells were then harvested by centrifugation at 5000*g* for 15 min at 4 °C. The cell pellet was resuspended in 20 mL of cold cell lysis buffer [PBS buffer (pH 7.4) containing 1 mM EDTA, and 0.1 mM DTT]. The cells were broken by sonication. The cell lysate was cleared by centrifugation at 10,000*g* for 20 min. The extract was slowly loaded onto the glutathione Sepharose 4B column which was equilibrated at 4 °C with column buffer [PBS buffer (pH 7.4) containing 1 mM EDTA, and 0.1 mM DTT]. After washing with column buffer until the OD_{280} reached less than 0.005, the 15-PGDH was eluted from the glutathione Sepharose 4B column by incubation at room temperature for 5 min with the elution buffer [50 mM Tris–HCl (pH 8.0) containing 10 mM reduced glutathione, 1 mM EDTA, and 0.1 mM DTT]. The concentration of the purified enzyme was determined and the purity of the enzyme was assessed by SDS–PAGE. The recombinant fusion enzyme exhibited a M_w of approximately 55 kDa.

3.5. 15-PGDH assay

The activity of 15-PGDH was assayed at 37 °C fluorometrically by measuring the formation of NADH at 468 nm following excitation at 340 nm using a Shimadzu RF-5301PC Fluorescence Spectrophotometer.[36] For determining the K_m value for prostaglandin E_2 (PGE_2), the reaction mixture contained: 50 mM Tris–HCl (pH 7.5), 0.1 mM DTT, 250 μM NAD^+, 20 μg of purified enzyme, and various concentrations of PGE_2 in 2 mL total volume. At least five different concentrations of PGE_2, ranging from 4 μM to 21 μM, and a fixed concentration of NAD^+ at 250 μM were used to determine K_m value for PGE_2. Each concentration was assayed in triplicate. For determining K_m value for NAD^+, PGE_2 at a final concentration of 21 μM was used with various concentrations of NAD^+ ranging from 25 μM to 250 μM. The spectrophotometer was standardized using a known concentration of NADH determined by measuring the absorbance at 340 nm using $\in_M = 6.22 \times 10^3\ M^{-1}\,cm^{-1}$.[37]

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