Structural and Mutational Analysis of *Trypanosoma brucei* Prostaglandin H₂ Reductase Provides Insight into the Catalytic Mechanism of Aldo-ketoreductases*

Kubata Bruno Kilunga,a,b,c,d Tsuyoshi Inoue,a,e,f Yousuke Okano,a,e,g Zakayi Kabututu,a,e,g Samuel K. Martin,b Michael Lazarus,a,b Michael Duszenko,c Yuichi Sumii,c Yukiko Kusakari,c Hiroyoshi Matsumura,c Yasushi Kai,c Shigeru Sugiyama,c Kouji Inaka,c Takashi Inui,a,b and Yoshihiro Uradea

From the aDepartment of Molecular Behavioral Biology, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan, Departments of Materials Chemistry and Molecular Protozoology, Osaka University, Yamada-oka, Suita, Osaka 565-0871, Japan, bUnited States Army Medical Research Unit-Kenya, Unit 64109, APO AE 09831-64109, cPhysiologisch-chemisches Institut der Universität Tübingen, Hoppe-Seyler-Strasse 4, 72076 Tübingen, Germany, dMARIWA Foods Industries Inc., 170 Tsutsu-cho, Yanatokoriyama, Nara 639-1123, Japan, eDepartment of Food and Nutrition, Tsu City College, 157 Ichihiden, Tsu City, Mie 514-0112, Japan, and fStructure and Function of Biomolecules Group, PRESTO, Japan Science Technology Agency, Honcho Kawanagashi, Saitama 332-0012, Japan

Aldo-ketoreductases are monomeric, NAD(P)H-dependent oxidoreductases present in nearly all organisms from prokaryotes (eubacteria and archaeabacteria) to eukaryotes (protozoa, vertebrates, invertebrates, plants, and fungi), in which they play important physiological roles. AKRs bind NAD(P)(H), but they do not possess the canonic Rossmann fold motif (1–4), and they metabolize a variety of substrates, including steroid hormones, monosaccharides, prostaglandins (PGs), isoflavonoids, and so forth (5–7).

Earlier structural and functional studies on AKRs have elucidated the catalytic mechanism of the oxidation/reduction reaction for physiological substrates such as monosaccharides (8–10), steroid hormones (4), and aldehydes (1, 11). These studies revealed that bacterial and mammalian AKRs catalyze the oxidation/reduction through a catalytic mechanism that involves a catalytic tetrad of aspartate, tyrosine, lysine, and histidine. Although the roles of these residues on catalysis of the oxidation/reduction reaction for a number of substrates have been elucidated, little is known about the way they affect the oxidation/reduction of prostaglandins. Nonetheless, the positional and evolutionary conservation of the residues of this catalytic tetrad in AKRs from different biological kingdoms would suggest that this catalytic mechanism is probably common to all AKRs.

PGF₂α is one of the earliest discovered prostanoids for which the synthetic pathways and biological functions have been investigated extensively. PGF₂α is synthesized by NADPH-dependent reduction catalyzed by AKRs of either the 9,11-endoperoxide moiety of PGH₂ or the 9-keto group of PGE₂ (12, 13). PGF₂α synthase (EC 1.1.1.188) was first isolated from mammals (14), and the mammalian enzymes, which belong to the 1C subfamily of the AKRs (see Refs. 15 and 16 for AKR superfamily classification), catalyze the reduction of PGH₂ to PGF₂α and PGG₂ to 9α,11β-PGF₂ (a stereoisomer of PGF₂α) (17), in addition to the oxidation of 9α,11β-PGF₂ to PGG₂ (18, 19). In mammals, PGF₂α is a potent mediator of various physiological processes (20–22) including regulation of vascular tone, constriction of uterine muscle (23) and pulmonary arter-

---

* This work was supported by Grants-in-aid for Scientific Research (B) 14370087 (to K. B. K. and T. I.), 14021130 (to K. B. K.), and 16017260 (to T. I.) from the Ministry of Education, Culture, Sport, Science and Technology, Japan; grants from PRESTO Project, Japan Science and Technology Agency (to T. I.); grants from Ono Medical Research Foundation (to K. B. K.); grants from ‘Applied Research Pilot Project for the Industrial Use of Space’ promoted by Japan Aerospace Exploration Agency and Japan Space Utilization Promotion Center (to Y. U.); grants from Handai Frontier Research Center (to T. I.); and by Japan Society for the Promotion of Science Postdoctoral Fellowship 02271 (to Z. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1VBJ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† These authors contributed equally to this work.

‡ To whom correspondence should be addressed: United States Army Medical Research Unit-Kenya, Unit 64109, APO AE 09831-64109. Tel.: 254-733-665210; Fax: 1-202-478-5084; E-mail: bkubata@nairobi.mimcom.net.

§ Present address: Dept. of Neurology, Beth Israel Deaconess Medical Center, Harvard University, 77 Ave. Louis Pasteur, Boston, MA 02115.

The abbreviations used are: AKR, aldo-ketoreductase; PG, prostaglandin; TpPGFS, *Trypanosoma brucei* prostaglandin F₂α synthase; 9,10-PQ, 9,10-phenanthrenequinone; 2,5DKG, 2,5-diketo-D-gluconic acid reductase A; ADR, aldose reductase; 3α-HSD, 3α-hydroxysteroid/dihydriodiol dehydrogenase.
ies (24, 25), and induction of luteolysis during the estrous cycle and prior to parturition (26, 27). During pathological processes in mammals, PGF2α overproduction causes ovarian dysfunction and miscarriage (28–30).

We have identified a unique PGF2α synthase from the protozoan parasite *Trypanosoma brucei* (31), the etiological agent of African trypanosomiasis in humans and animals. *T. brucei* PGF2α synthase belongs to 5A subfamily of the AKRs (16, 31). The uniqueness of this synthase stems from the discovery that it exhibits a high specificity toward PGH2 and shows no enzymatic activity toward substrates such as PGD2, PGE2, or PGF2α. TbPGFS catalyzes the NADPH-dependent reduction of PGH2 to PGF2α, in addition to the reduction of 9,10-PQ, with the lowest Km value for PGH2 among the various AKRs. Although it has been shown that PGF2α elicits vacuole formation and thus may play the signal coupling role during phagocytosis in the protozoan parasite *Amoeba proteus* (32), little is known to why *T. brucei* produces PGF2α. However, because African trypanosomiasis is characterized by miscarriage due to PGF2α overproduction correlated with parasitemia peaks (29), findings that *T. brucei* possesses a TbPGFS suggest that this enzyme may well play a role in the pathogenesis of trypanosomiasis. Hence, the elucidation of TbPGFS structure and mechanism of catalysis is of importance because it may lead to the design of specific inhibitors needed for investigating the physiological role(s) of the enzyme in this organism.

Here, we describe the x-ray crystallographic structure of the TbPGFS-NADPH−citrate ternary complex at 2.1 Å resolution. TbPGFS structure showed the positional and evolutionary conservation of Asp77, Tyr52, Lys77, and His110 in the catalytic pocket. Although the tyrosine residue has been essential for catalysis in most AKRs, our mutagenesis experiments on the catalytic tetrad revealed that TbPGFS required Lys77 and His110 but not Tyr52 for the biological reduction of PGH2 to PGF2α, whereas it needed Lys77, Tyr52, and His110 for the reduction of 9,10-PQ. Thus, we propose a catalytic mechanism by which a catalytic dyad of Lys77 and His110 in TbPGFS is involved in proton transfer to the dehydrogenase subunit.

**Site-directed Mutagenesis—**For point mutations of TbPGFS, highly conserved amino acid residues to be mutated were selected based on the detailed structural information of the TbPGFS-NADPH−citrate ternary complex. Polymerase chain reaction mutagenesis was carried out to generate the appropriate mutated cDNAs. A series of sense (F) and antisense (R) primers containing mismatched codons for each mutant were designed for PCR mutagenesis. Site-directed mutagenesis was used to produce single (D47N, Y52F, K77R, H110A, and H110F), double (D47N/H110A, Y52F/K77L, Y52F/H110A, and K77L/H110A), and triple (Y52F/K77L/H110A) mutants. The single mutant enzymes were used to obtain the catalytic tetrad. The resultant recombinant vectors were used for transformation of *Escherichia coli* BL21(DE3). Transformed cells were cultured for 8–10 h in the presence of 0.5 mM isopropyl-β-D-thiogalactopyranoside at 30 °C. The resultant recombinant enzymes were purified by centrifugation; washed with phosphate-buffered saline containing a mixture of 100 mM sodium phosphate (pH 7.0), and 500 μM 9,10-PQ, in a total volume of 500 μl. The purified enzymes were dialyzed against 20 mM Tris/Cl (pH 8.0) buffer and applied to a DEAE anion-exchange column that had been equilibrated with the same buffer. The proteins were eluted with phosphate-buffered saline. The purity of the proteins was assessed by SDS-PAGE on 14% (w/v) gels, and the gels were stained with Sypro Orange (Bio-Rad) or Coomassie Brilliant Blue (Daichi Pure Chemicals).

**Enzyme Assay and Kinetic Measurements—**PGH2 reductase activity was measured by incubating at 37 °C for 2 min a reaction mixture containing an appropriate enzyme aliquot, 5 μM [1-14C]PGH2 (final concentration), 100 μM of 100 mM sodium phosphate (pH 7.0), and 500 μM 9,10-PQ at 30 °C. After the reaction had been terminated by the addition of 250 μl of stop solution (30:1 (v/v) diethyl ether/methanol/2 M citric acid), aliquots were analyzed by thin-layer chromatography as described previously (31). For the reduction of 9,10-PQ, the reaction mixture consisted of 100 mM sodium phosphate (pH 7.0), the enzyme, 100 μM NADPH, and 40 μM 9,10-PQ, in a total volume of 500 μl. The reaction was initiated by the addition of the substrate, and then the decrease in absorbance at 340 nm (ε = 6270 M−1 cm−1) was monitored.
**RESULTS AND DISCUSSION**

We determined the structure of the TbPGFS-NADP$^+$-citrate ternary complex by molecular replacement at 2.1 Å resolution with $R_{\text{cryst}}$ and $R_{\text{free}}$ values of 23.1 and 26.8%, respectively. Other crystallographic properties and refinement statistics of the structure are shown in Table I.

TbPGFS is composed of a cylindrical core of eight parallel $\beta$-strands surrounded by 8 $\alpha$-helices running antiparallel to the $\beta$-sheets (Fig. 1, A and B). This $\alpha/\beta$_8_ structure is interrupted by two extra $\alpha$-helices (H1 and H2) situated toward the COOH-terminal side of the molecule. The helix H1 lies in between loops 7–9 and 14–16, joined by a tight turn. These extra structures, i.e., H1, H2, B1, and B2, have also been described in other members of the AKR superfamily (2, 10, 39). The COOH-terminal 19 amino acid residues sketch out the upper part of the substrate-binding pocket by constructing a lip-like structure around the edge of the COOH-terminal face of the central $\beta$-barrel. The overall structure of TbPGFS is homologous to that of Corynebacterium 2,5-diketo-d-glucuronic acid reductase A (2,5DKG) (8, 16), both of which belong to the fifth subfamily of the AKR superfamily (Fig. 1B). It is also essentially similar to the structures of members of the first subfamily of AKRs, including human aldose reductase (ADR) holoenzyme (2), porcine ADR (10), and rat liver 3α-hydroxysteroid/dihydrodiol dehydrogenase (3α-HSD) (4). However, one of the major structural differences between TbPGFS and members of the first subfamily of AKRs is the presence of shorter loops in the structure of TbPGFS. A superimposition, with a root mean square deviation of 1.5 Å from the corresponding C$^\alpha$ atoms, of the TbPGFS model on the rat 3α-HSD (Fig. 1C) showed deletions in loops $\beta_1$-$\alpha_1$, $\beta_4$-$\alpha_4$, and $\beta_7$-$\alpha_1$ in TbPGFS in contrast to the presence of significantly longer loops in enzymes of the first subfamily of AKRs. As a result, TbPGFS possesses shallower cofactor- and substrate-binding sites, whereas rat 3α-HSD shows a deep corresponding site created by the longer loops. The presence of short loops in TbPGFS and Corynebacterium 2,5DKG may thus be a common characteristic of the members of the fifth subfamily of AKRs.

TbPGFS does not possess the Rossmann fold for binding NADPH. The NADP$^+$ pyridine nucleotide is bound in an extended conformation within a crevice that extends from the outer edge of the barrel to the inner core. The nicotinamide ring is positioned deep in the central region of the $\beta$-barrel. The NADP$^+$ adenine and pyrophosphate groups are wedged on the surface of the depression between the $\alpha_7$ and $\alpha_8$ helices (Fig. 1B). The electron density map of the NADP$^+$ molecule bound to TbPGFS (Fig. 2A) showed that the enzyme-NADP$^+$ complex was stabilized by 16 hydrogen and 3 ionic bonds and 1 aromatic stacking interaction (Fig. 2B). The number of interactions is lower than that for AKRs of the first subfamily, such as human ADR holoenzyme and rat 3α-HSD, both of which have 19 hydrogen bonds, 3 salt bridges, and 1 aromatic stacking (2, 4). Fluorescence quenching assay (data not shown) revealed that the $K_q$ value of TbPGFS for NADPH (5.3 $\mu$M) was 1 order of magnitude higher than that of rat 3α-HSD, i.e., 143 nM (40), indicating a relatively loose binding of NADPH in TbPGFS as compared with the tight binding in AKRs of the first subfamily. The weaker affinity for NADPH may be responsible, in part, for the 3-fold higher $K_m$ value of 5.7 $\mu$M (31) for TbPGFS compared with 2 $\mu$M for human ADR holoenzyme (10). In TbPGFS, the pyrophosphate group of NADP$^+$ is bound in a crevice on the surface of the barrel. On one side, the crevice lines Ser$^{188}$, Leu$^{190}$, and Gln$^{192}$, all provided by loop $\beta_7$-$\alpha_1$. On the other side, it has only Lys$^{230}$, held by loop $\beta_8$-$\alpha_8$. The pyrophosphate group is held in place by hydrogen bonding interactions with Ser$^{188}$, Leu$^{190}$, Gln$^{192}$, and one molecule of water in one side and Lys$^{230}$ in the other side (Fig. 2, A and B). In contrast, in the AKRs of the first subfamily, the extended polypeptide region of loop $\beta_7$-$\alpha_1$ folds over the pyrophosphate group of NADPH and builds a short tunnel, known as the "safety belt," through which the pyrophosphate moiety passes. In human ADR holoenzyme, salt bridges between Asp$^{726}$, Lys$^{721}$, and Lys$^{262}$ stabilize this safety belt (2). However, the safety belt is absent in TbPGFS, and Asp$^{726}$ has been deleted from loop $\beta_7$-$\alpha_1$. Lys$^{724}$ (similar to Lys$^{231}$ in human ADR holoenzyme) is located too far, i.e., ~6.3 Å, from the pyrophosphate group. Although the salt linkage between the pyrophosphate group and Lys$^{262}$ has been conserved in the structure of TbPGFS, the lack of the safety belt may account for the high $K_q$ value of NADPH observed for TbPGFS. In addition, AKR members have conserved most of the residues involved in NADP$^+$ pyridine nucleotide binding. However, the involvement of Met$^{22}$, Trp$^{187}$, Gln$^{192}$, and Gly$^{232}$ in NADP$^+$ binding appears to be
FIG. 1. A, structural alignment of TbPGFS (AKR5A2) with several members of the AKR superfamily. Alignments were calculated with the COMPARER program (51) using Protein Data Bank entries 1A80, 2ACQ, 1AH3, and 1AFS for Corynebacterium 2,5DKG (AKR5C), human ADR holoenzyme (AKR1B1), porcine ADR (AKR1B6), and rat 3α-HSD (AKR1C9), respectively. The secondary structure of TbPGFS is indicated above the
unique for TbPGFS. We also found that in TbPGFS structure, the amido atom of 3-carboxyamidloye side chain of the NADP⁺ ring hydrogen bonds Ser130 and Gln161, both of which are conserved among the AKR members (Fig. 1A). Trp187 indole ring is involved in a π–π interaction with the NADP⁺ ring. In contrast, in human ADR holoenzyme, porcine ADR, and rat 3α-HSD, Tyr that has substituted Trp187 maintains the π–π interaction with the NADP⁺ ring. On the other hand, phosphate group of 2′-AMP is hydrogen-bonded to Lys53 and Arg236 of TbPGFS, two residues that are conserved among AKRs (with the exception of rat 3α-HSD, in which Lys230 has been substituted by Arg236). Moreover, with regard to the 2′-AMP phosphate group binding, a unique feature of TbPGFS is that the amido nitrogen atom of Gly235 (a residue that is not conserved among AKRs) also hydrogen bonds the phosphate group. However, because the amido nitrogen atom of the main chain forms this interaction, the phosphate group bonds to either Val232 in Corynebacterium 2.5DKG, human ADR holoenzyme, and porcine ADR or Phe232 in rat 3α-HSD.

The active site of TbPGFS is located at the COOH-terminal end of the β-barrel (Fig. 3a–e), as in the case of all other αβ-barrel enzymes (39, 41). Within the active site pocket, the nicotinamide ring is oriented so that the 4-pro-R hydrogen is directed up toward the pocket opening. The TbPGFS active site pocket has the dimensions of 3 x 5 x 7 Å (length x width x depth) from the nicotinamide ring C₆ position to the upper surface of the TbPGFS molecule (Fig. 3a). These dimensions of the TbPGFS pocket are significantly smaller when compared with 5 x 6 x 9 Å (Fig. 3b) for Corynebacterium 2.5DKG (8) or 7 x 13 x 10 Å (Fig. 3c) for human ADR holoenzyme (2). In TbPGFS, Met238, Asp47, Ala49, Lys77, Ser130, Asn140, Gln161, and Trp187 are invariant in the bottom part of this pocket. The upper side of the pocket is composed of aromatic and apolar residues including Trp49, Ile51, Tyr52, Trp79, His110, and Trp187. In addition, we used citrate (an inhibitor of the reduction of PGH₂ by TbPGFS) in co-crystallization experiments in order to get insight into its binding to the enzyme. Fig. 4A shows a TbPGFS/NADP⁺–citrate ternary structure with the electron density map of the bound citrate molecule. In this ternary complex structure, citrate was located above the bound NADP⁺ with its carboxyl O₁ penetrates deep into the substrate-binding site. This carboxyl O₁ is hydrogen-bonded to the O’ atom of Tyr52 and the N’ atom of His110 at a distance of 2.76 and 2.54 Å, respectively (Fig. 4B), whereas Asp47 and Lys77 are located far from the citrate, at a distance of 6.61 and 4.98 Å, respectively. Hydrogen bondings similar to those between Tyr52–substrate–His110 (in TbPGFS) were also identified in the recently determined human PGFS (3α-HSD)/PGD₂-NADP⁺ ternary complex structure (42), in which Tyr52 and His110 correspond to Tyr52 and His110 of TbPGFS. Furthermore, the carboxyl O₁ of citrate is located at a distance of 3.30 Å to C₆ of NADP⁺.

A number of highly conserved amino acid residues have been identified in the close vicinity of the TbPGFS nicotinamide ring in the active site pocket of AKRs. A comparison of the structure of TbPGFS with the structures of Corynebacterium 2.5DKG, human ADR holoenzyme, porcine ADR, and rat 3α-HSD identified Asp47, Ala49, Tyr52, Lys77, Trp79, and His110 as being those highly conserved amino acid residues in TbPGFS, whereas the amino acids at position 51, which is known to determine the specificity of substrates in AKRs, are highly divergent. The crystal structure of Corynebacterium 2.5DKG contains two well-ordered water molecules (Fig. 3b) that play an important role in the orientation of the carbonyl and hydroxyl groups of the substrate within the catalytic pocket (43). However, instead of water, TbPGFS crystal structure contained one citrate molecule (Fig. 3a). A superimposition of NADP⁺ molecules bound to TbPGFS with those bound to human ADR holoenzyme (2), porcine ADR (44), and rat 3α-HSD (4) with a root mean square deviation of 0.17, 0.20, and 0.24 Å, respectively, revealed that in these enzymes the substrate-binding sites corresponded to the site of citrate binding, thus identifying this site as the putative substrate-binding site.

Most AKRs catalyze the oxidation/reduction of both physiological and synthetic (9,10-PQ) substrates by a mechanism thought to be common for all members of the superfamily. To elucidate the mode of action of TbPGFS on PGH₂ and 9,10-PQ reduction, we generated a number of single, double, and triple mutants in the residues of the catalytic tetrad and assessed the effect of each mutation on the reduction of both substrates. Mutants K77L and H110F completely abolished their PGH₂ reductase activity (Fig. 5A, left panel and inset), whereas mutants Y52F, K77L, H110A, and H110F were inactive on 9,10-PQ (Fig. 5B, left panel). Replacement of Leu77 by a positively charged residue in L77R mutant restored 60% and 52% of the reductase activity toward PGH₂ and 9,10-PQ, respectively. In contrast, H110A mutation produced a mutant that exhibited 2.9% of PGH₂ reductase activity as compared with the wild-type enzyme. However, this same mutation resulted in inactive mutant on 9,10-PQ (Fig. 5, A and B, left panels). Unexpectedly, none of the other single mutations altered the PGH₂ reductase activity, indicating that in TbPGFS, a diad of Lys77 and His110, but not a tetrad of Asp47, Tyr52, Lys77, and His110, was essential for the PGH₂ reductase activity. Mutants Y52F/H110A and D47N/H110A retained some of their reductase activity toward PGH₂, whereas Y52F/K77L, K77L/H110A, and Y52F/K77L/H110A completely lost this activity (data not shown). None of these double and triple mutants exhibited 9,10-PQ reductase activity. These results suggest that TbPGFS uses two independent mechanisms to catalyze the reduction of both PGH₂ and 9,10-PQ. The reduction of PGH₂ to PGF₂α appears to be achieved by a catalytically essential diad of Lys77 and His110, but not Tyr52 and/or Asp47, whereas a catalytic triad of Tyr52, Lys77, and His110 is needed for the reduction of the non-physiological substrate 9,10-PQ. Previous mutagenesis studies have shown that the Y55F mutant of rat liver 3α-HSD (an AKRC1C9) abolished the reductase activity toward steroid substrates (45) but retained the reduction of 9,10-PQ (46). In the present study, we show that Y52F mutant of TbPGFS (an AKR5A2) retained the reductase activity toward the physiological substrate (PGH₂), while abolishing the reduction of the non-physiological substrate 9,10-PQ. Instead, TbPGFS used a distinct residue (His110) to achieve the reduction of PGH₂. Although the presence of two different catalytic mechanisms for the reduction of two different substrates within the same
active site of AKRs has been reported (45, 46), our findings show that at least TbPGFS catalyzes the reduction of PGH₂ through a different mechanism and that the non-physiological substrate 9,10-PQ appears to be reduced by a mechanism similar to that of steroid reduction by rat 3α-HSD, in which the Tyr residue is essential.

Kinetic analysis of the mutants (Table II) revealed that the catalytic efficiency, $k_{\text{cat}}/K_m$ (PGH₂), of the invariable catalytic tyrosine mutant (Y52F) was similar to that of the wild-type enzyme, proving that neither $k_{\text{cat}}$ nor $K_m$ (PGH₂) was significantly affected by this mutation. H110A mutant showed a 35-fold decrease in $k_{\text{cat}}$ and a 5-fold increase in $K_m$ (PGH₂) that resulted in a 183-fold decrease in the $k_{\text{cat}}/K_m$ (PGH₂) value. On the other hand, D47N and K77R mutants exhibited an increased $k_{\text{cat}}$ (almost 1.6-fold) and $K_m$ for PGH₂ (10- and 20-fold, respectively), leading to a significant decrease in their $k_{\text{cat}}/K_m$ values. In contrast, these same mutants (D47N and K77R) showed a decreased $k_{\text{cat}}$ (almost 1.4- and 1.9-fold, respectively).
and an increased $K_m$ for 9,10-PQ (47- and 16-fold, respectively), which resulted in a very low catalytic efficiency. Because structural analysis revealed hydrogen bonds between Asp47-NADP/H11001 (Fig. 2, A and B) and Asp47-Lys77 (Fig. 6B), it is most likely that D47N mutation produced a mutant with a weak hydrogen bond between Asn47-Lys77 and low affinity between Asn47-NADPH. This change could well affect substrate binding and the catalytic efficiency for the reduction of both substrates by this mutant. On the other hand, the increment of $K_m$ for PGH2 and 9,10-PQ in K77R may be due to a change in substrate binding properties as a result of Arg77 rigidity.

The loss of activity observed throughout this study could...
come from a conformational change rather than from any specific effect of the particular mutation on catalysis. CD spectra of the wild-type and mutant enzymes did not suggest any significant alteration of the ternary structure in the mutant proteins (data not shown), suggesting that these mutations did not result in any significant change in the overall three-dimensional structures. In addition, wild-type TbPGFS and its inactive mutants (Y52F, K77L, H110A, and H110F) did bind NADPH (data not shown) with a binding constant \( K_d \) that was almost similar to that of the wild-type enzyme (5.3 \( \mu \)M), confirming that mutations did not affect the overall three-dimensional structures of the resulting mutants. We tested also the effect of PGH2 analogs (U-44069 and U-46619) on the reduction of PGH2 by wild-type TbPGFS. Various concentrations of homologs were pre-incubated with TbPGFS (1–2 \( \mu \)g) at 37 °C for 2 min, followed by incubation of the enzyme with 5 \( \mu \)M [1-\( ^{14} \)C]PGH2 in the presence of NADPH. The residual enzymatic activity was plotted against PGH2 homolog concentrations.

**Fig. 5.** A, left panel, specific activities of wild-type and mutant enzymes. Pure recombinant wild-type (Wt) or mutants of TbPGFS (1–2 \( \mu \)g) were incubated with 5 \( \mu \)M [1-\( ^{14} \)C]PGH2 in the presence of NADPH as described under “Materials and Methods.” PGH2 conversion to PGF2\(_{\alpha}\) was qualitatively (inset) and quantitatively (the bar graph) analyzed by thin-layer chromatography and autoradiography as shown in the inset, and the corresponding values of the spot densities were plotted. Right panel, pH dependence of PGH2 reduction by wild-type and mutant TbPGFS. B, left panel, NADPH-dependent reduction of 9,10-PQ by wild-type and mutant TbPGFS. Experimental details are described under “Materials and Methods.” Right panel, pH dependence of 9,10-PQ reduction by wild-type and mutant TbPGFS. C, schematic drawing of PGH2 and PGH2 homologs U-44069 and U-46619. D, effect of U-46619 and U-44069 on the reduction of PGH2 by wild-type TbPGFS. Various concentrations of homologs were pre-incubated with TbPGFS (1–2 \( \mu \)g) at 37 °C for 2 min, followed by incubation of the enzyme with 5 \( \mu \)M [1-\( ^{14} \)C]PGH2 in the presence of NADPH.
substituted with a CH₂ group (Fig. 5C). Pre-incubation of wild-type TbPGFS with various concentrations of U-44069 had no effect on the reduction of both PGH₂ (Fig. 5D) and 9,10-PQ (data not shown). In contrast, treatment with U-46619 partially inhibited the reduction of PGH₂ by TbPGFS in a dose-dependent manner (Fig. 5D), but it had no effect on the reduction of 9,10-PQ (data not shown). These findings provide evidence of the need for free oxygen at the C₁₁ position of PGH₂ for the substrate to enter the catalytic pocket and bind to TbPGFS.

We constructed a PGH₂ binding model (Fig. 6) based on the electrostatic potential on the surface of TbPGFS, the details of citrate binding to TbPGFS, and the kinetics of PGH₂ analogs binding to the wild-type enzyme. The energy minimization of the PGH₂ binding model was achieved by using the CNS program. In this model, PGH₂ binds above the nicotinamide ring of the cofactor in an extended conformation across the shallow elliptical crevice (Fig. 6A). PGH₂ chains α and ω extend in opposite directions and straddle the lip of the crevice. This orientation is justified by the analysis of the electrostatic potential surface model of PGH₂, which revealed that α-chain of the substrate would interact with the only basic residue, Lys⁸⁷, and that ω-chain may interact with the hydrophobic residue Trp²³. The hydroxyl group in the ω-chain of PGH₂ is located at a distance of 2.72 Å from the N^+ atom of Trp²³, suggesting that Trp²³ may well recognize the ω-chain as evidenced in the TbPGFS-NAD⁺·citrate ternary structure, where the carboxyl O₅ of citrate was hydrogen-bonded to the N^+ atom of Trp²³ at a distance of 3.44 Å (Fig. 4B). No basic residues were found in the putative binding site around the ω-chain of PGH₂. The cyclopentane ring of PGH₂, with its 9,11-endoperoxide bond, is oriented deep in the cavity in the vicinity of the nicotinamide ring of NAD⁺, positioning the C₉ oxygen atom of PGH₂ at 3.3 Å from the C₁ of the nicotinamide ring (Fig. 6B). On the other hand, Lys⁷⁷ and His¹¹⁰ are located at 7.69 and 2.62 Å, respectively, from the C₁ oxygen of PGH₂. These geometrical arrangements indicate that His¹¹⁰, but not Lys⁷⁷, is in close vicinity to C₁₁ oxygen of PGH₂ and therefore may easily be involved in hydrogen bonding of the substrate and proton transfer during the reduction of PGH₂. More recently, a ternary structure of human PGFS-NAD⁺·PGD₂ was reported (42). The PGH₂ binding model we propose here is in agreement with PGD₂ binding to the human PGFS (3α-HSD, an enzyme that catalyzes the reduction of PGH₂ to PGF₂α, the reduction of PGD₂ to 9α,11β-PGF₂α, and the oxidation of 9α,11β-PGF₂α to PGD₂) because the ternary structure of human 3α-HSD-NAD⁺·PGD₂ revealed that the cyclopentane ring of PGD₂ (a prostaglandin that possesses a cyclopentane ring as PGH₂) was oriented deep within the active site cavity, whereas α- and ω-chains of PGD₂ remained far from the active site cavity and showed little interaction with the enzyme.

Mutagenesis and kinetic studies of AKRs revealed that bacterial and mammalian enzymes catalyze the oxidation/reduction reactions through a mechanism that involves a catalytic tetrad of tyrosine, histidine, lysine, and aspartate (1–4, 8, 44, 45, 47, 48). Tyrosine acts as the general acid/base catalyst. Histidine facilitates proton donation during reduction (46) or orients the substrate carbonyl in the active site (9, 43), whereas lysine helps proton removal by tyrosine during oxidation by making a hydrogen bond that lowers the pKₐ value of the tyrosine. Aspartate forms a salt bridge to stabilize lysine (3, 4, 40). In order to catalyze the oxidation/reduction reaction according to this mechanism, tyrosine OH has been involved in hydrogen bond networks with histidine and lysine. In the present study, we found that among the residues of the catalytic tetrad of TbPGFS, Lys⁷⁷ and His¹¹⁰ are catalytically important for the reduction of PGH₂ whereas Tyr⁵², Lys⁷⁷, and His¹¹⁰ are essential in the reduction of 9,10-PQ. Observations that mutation of Tyr⁵², the putative proton donor in most of AKRs, led to a retention of PGH₂ reductase activity but a loss of 9,10-PQ reductase activity prompted a thorough investigation of the effect of pH on catalysis in an effort to assign the titratable groups involved in the reduction of these two substrates. The pKₐ and pH-independent values of kₐₙ for the wild-type and mutant enzymes are summarized in Table III. The log kₐₙ value for the reduction of PGH₂ by wild-type TbPGFS (Fig. 5A, right panel) increased with the increase of pH up to pH 6.5 and then decreased with the increase of pH, showing an ionizable group with a pKₐ value of 7.4 ± 0.08 (Table III) that should be protonated for maximal activity. A comparison of the log kₐₙ versus pH profiles for the tetrad mutants revealed the presence in D47N, Y52F, and K77R mutants of a titratable group with a pKₐ value of 7.80, 7.30, and 7.40, respectively, that must be protonated for maximal PGH₂ reduction. However, this titratable group is not observed in mutants K77L and H110F. In addition, the log kₐₙ versus pH profiles also show a titratable group with a lower pKₐ value of 5.8 ± 0.1 for H110A mutant (that exhibited trace of PGH₂ reduction) that must be protonated for maximal PGH₂ reduction. Although Lys⁷⁷ and His¹¹⁰ can each act as a proton donor, structural data revealed that Lys⁷⁷ is located too far (6.35 Å) from the C₁ of the nicotinamide ring (Fig. 6B) and thus would probably not act as a proton donor for PGH₂. Taken together, these data suggest that His¹¹⁰ (pKₐ = 5–8 in proteins) but not Lys⁷⁷ (pKₐ = ~10) plays a dominant role in catalysis and thus may be the titratable group responsible for PGH₂ reduction. In contrast, the kₐₙ versus pH profiles for the reduction of 9,10-PQ by wild-type TbPGFS (Fig. 5B, right panel) showed a kₐₙ value that increases with increasing pH up to pH 8.0 and then falls with increasing pH, revealing the presence of a titratable group with a pKₐ value of 8.5 ± 0.05 that must be protonated for maximal activity. The 1.1-pH unit difference of the pKₐ value between the reduction of PGH₂ and 9,10-PQ suggests that two different ionizable groups might be involved in the reduction of the two different

| Enzyme      | PGH₂ | 9,10-PQ |
|-------------|------|---------|
|             | kₐₙ | Kₐₙ | kₐₙ/Kₐₙ | kₐₙ | Kₐₙ | kₐₙ/Kₐₙ |
| Wild-type   | 76   | 1.8  | 42.0    | 1467 | 0.45 | 3260    |
| D47N        | 122  | 19.0 | 6.2     | 1048 | 21   | 50      |
| Y52F        | 79   | 1.7  | 46.0    | ND   | ND   | ND      |
| K77L        | ND   | ND   | ND      | ND   | ND   | ND      |
| K77R        | 120  | 36.0 | 3.3     | 758  | 7    | 108     |
| H110A       | 2.2  | 9.7  | 0.23    | ND   | ND   | ND      |
| H110F       | ND   | ND   | ND      | ND   | ND   | ND      |

*ND, not determined.

The limit of detection for PGH₂ reductase assay was 0.8 μmol/min/μmol protein.
Fig. 6. A, a proposed model of PGH₂ binding to TbPGFS. The electrostatic potential of the surface of TbPGFS and the cofactor binding model show the PGH₂ binding pocket of this enzyme (left panel). A close-up view (right panel) gives details on how PGH₂ (drawn in green) fits the substrate-binding cleft. The cyclopentane ring of PGH₂ moves into the vicinity of the C₄ of the nicotinamide ring of NADPH (shown as a ball-and-stick model), and then the α- and ω-chains of PGH₂ lie opposite each other within the elongated binding cleft. The rationale used for the catalytic mechanism of 9,11-endoperoxide PGH₂ reductase.
the PGH₂ binding model is described under “Results and Discussion.” The PGH₂ binding model was made with the programs GRASP, MOLSCRIPT, and RASTER3D; colored according to the calculated electrostatic potentials; and contoured from 1.6 pH unit between the wild-type and H110A mutant. A profile of log $k_{\text{cat}}$ versus pH plot is shallow with a slope $< 1.0$ may suggest that that the titratable group is not a single ionizable amino acid but rather a group of residues, i.e. the Lys²²7 and His³¹⁰ dyad. Furthermore, TbPGFS appears to be a rare example of an AKR that uses histidine to stabilize its proton (H⁺, red) to C₁₁ carbonyl (δ⁻) of the endoperoxide. In addition, through a water molecule bound between its carboxyl group and the N⁵¹ atom of the imidazole ring of His³¹⁰, this positively charged residue would facilitate His³¹⁰ protonation. This assumption is supported by the fact that a mutation of the positively charged residue Lys²²⁷ to a hydrophobic amino acid, leucine, led to a complete loss of activity by the mutant K₇⁷L and that this lost activity was restored by replacement of Leu¹⁷ by arginine (another positively charged residue) in L⁷⁷R mutant. Our results therefore show the importance of Lys²²⁷ in facilitating the spatial arrangement via an electrostatic repulsion against His³¹⁰ and protonation of His³¹⁰ through a water molecule, in addition to forming a salt bridge to stabilize Asp⁶⁷.

We have successfully solved the three-dimensional structure of TbPGFS that was co-crystallized in the presence of NADP⁺ and citrate. Our x-ray data provided structural details of NADP⁺ and citrate binding that were used for the modeling of PGH₂ binding to the enzyme. The presence of only two and three catalytic residues for the reduction of PGH₂ and 9,10-PQ instead of four was confirmed by mutagenesis and kinetic studies. Our results provide the first insights into the mechanism of the biological reduction of PGH₂ by a prostaglandin F₂α, synthase and the critical role of histidine, but not tyrosine, in catalysis. We therefore propose a novel catalytic mechanism for the reduction of PGH₂ to PGF₂α that involves a dyad of Lys⁷⁷ and His¹¹⁰ in which His¹¹⁰ acts as a general acid catalyst.

Acknowledgments—We are grateful to M. Tang of SPring-8 beamline 12B2 for the fundamental data collection. We also thank Dr. H. Toh and H. Ichihara for amino acid sequence alignment. We are grateful to Dr. C. J. Mundy for data collection and Dr. T. Okada for assistance in DNA sequence analysis.

REFERENCES

1. El-Kabbani, O., Judge, K., Ginell, S. L., Myles, D. A., DeLucas, L. J., and Flynn, T. G. (1995) Nat. Struct. Biol. 2, 687–692

---

### Table III

| Enzyme | Substrate | $pK_a$ | $C^\text{v}$ |
|--------|-----------|--------|-------------|
| Wild-type | PGH₂ | 7.40 ± 0.08 | 0.04 |
| 9,10-PQ | 8.50 ± 0.05 | 126 ± 0.5 |
| D47N | PGH₂ | 7.80 ± 0.14 | 195.3 ± 15 |
| 9,10-PQ | 8.00 ± 0.2 | 1897 ± 2.42 |
| Y52F | PGH₂ | 7.30 ± 0.11 | 115 ± 0.6 |
| 9,10-PQ | 5.8 ± 0.10 | 1.2 ± 0.04 |
| K77R | PGH₂ | 7.40 ± 0.19 | 188 ± 0.10 |
| 9,10-PQ | 8.30 ± 0.40 | 1816 ± 3.13 |
| H110A | PGH₂ | 5.80 ± 0.19 | 188 ± 0.10 |
| 9,10-PQ | 8.30 ± 0.40 | 1816 ± 3.13 |

$^a$ pH independent values of $k_{\text{cat}}$ are expressed in units of min⁻¹.

---

substrates. This finding is consistent with our site-directed mutagenesis data and explains our previous results showing that the reduction of PGH₂ by TbPGFS was not inhibited by 9,10-PQ when both substrates were incubated simultaneously in excess (31). A profile of the $k_{\text{cat}}$ versus pH for the tetrad mutants revealed that mutants Y52F, K77L, H110A, and H110F eliminated the titratable group for 9,10-PQ reduction. Residues Tyr⁵², Lys⁷⁷, and His³¹⁰ can each act as a proton donor. However, Lys⁷⁷ will not be a proton donor because it is located too far away. Because the pH dependence of $k_{\text{cat}}$ for (wild-type) identified an ionizable group with a $pK_a$ value of 8.5 ± 0.05 that must be protonated for maximal activity, the ionizable residue responsible for 9,10-PQ reduction appears to be Tyr⁵². Tyrosine usually range of $pK_a$ in proteins is 9–12. In TbPGFS, the $pK_a$ value of 8.5 ± 0.05 appears to be lowered by hydrogen bond with Lys⁷⁷. Our mutagenesis and kinetic studies also show that the H110A mutant exhibited 2.9% of the PGH₂ reductase activity relative to wild-type enzyme, whereas the H110A mutant revealed that mutants Y52F, K77L, H110A, and H110F eliminated the titratable group for 9,10-PQ reduction. In addition, through a water molecule bound between its carboxyl group and the N⁵¹ atom of the imidazole ring of His³¹⁰, this positively charged residue would facilitate His³¹⁰ protonation. This assumption is supported by the fact that a mutation of the positively charged residue Lys⁷⁷ to a hydrophobic amino acid, leucine, led to a complete loss of activity by the mutant K77L and that this lost activity was restored by replacement of Leu¹⁷ by arginine (another positively charged residue) in L⁷⁷R mutant. Our results therefore show the importance of Lys⁷⁷ in facilitating the spatial arrangement via an electrostatic repulsion against His³¹⁰ and protonation of His³¹⁰ through a water molecule, in addition to forming a salt bridge to stabilize Asp⁶⁷.
Catalytic Mechanism of 9,11-Endoperoxide PGH2 Reductase

2. Wilson, D. K., Bohren, K. M., Gabbay, K. H., and Quiocho, F. A. (1992) Science 257, 81–84
3. Wilson, D. K., Nakano, T., Petrash, J. M., and Quiocho, F. A. (1995) Biochemistry 34, 14323–14330
4. Hoog, S. S., Pawlowski, J. E., Alzari, P. M., Penning, T. M., and Lewis, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2517–2521
5. Penning, T. M., Pawlowski, J. E., Schlegel, B. P., Jez, J. M., Lin, H. K., Hoog, S. S., Bennett, M. J., and Lewis, M. (1996) Steroids 61, 508–523
6. Petrash, J. M., Tarle, I., Wilson, D. K., and Quiocho, F. A. (1994) Diabetes 43, 955–969
7. Welle, R., Schroder, G., Schiltz, E., Grisebach, H., and Schroder, J. (1991) Eur. J. Biochem. 196, 423–430
8. Khurana, S., Powers, D. B., Anderson, S., and Blaber, M. (1998) Biochemistry 37, 606–611
9. Kubata, B. K., Duszenko, M., Kabututu, Z., Rawer, M., Szallies, A., Fujimori, K., Inui, T., Nozaki, T., Yamashita, K., Horii, T., Urade, Y., and Hayaishi, O. (2000) J. Exp. Med. 192, 1327–1338
10. Pruscho, R. D., Goette, S. M., and Haberman, P. (1989) Cell Tissue Res. 255, 533–537
11. Okano, Y., Inoue, T., Kubata, B. K., Kabututu, Z., Urade, Y., Matsumura, H., and Kai, Y. (2002) J. Biochem. (Tokyo) 132, 859–861
12. Navaza, J. (2001) Acta Crystallogr. Sect. D Biol. Crystallogr. 57, 1367–1372
13. Roussel, A., and Cambiulli, C. (1996) Protein Eng. 2188–2198
14. De Winter, H. L., and von Itzstein, M. (1995) Biochemistry 34, 24841–24847
15. Barski, O. A., Gabbay, K. H., Grinshaw, C. E., and Bohren, K. M. (1995) Biochemistry 34, 11264–11275
16. Watanabe, K., Yoshida, K., Shimizu, T., and Hayaishi, O. (1985) J. Biol. Chem. 260, 7035–7041
17. Jez, J. M., Flynn, T. G., and Penning, T. M. (1997) Biochemistry 36, 659–664
18. Suzuki, T., Fuji, Y., Miyano, M., Chen, L. Y., Takahashi, T., and Watanabe, K. (1996) J. Biol. Chem. 271, 24744–24750
19. Matsuzuru, K., Shiraishi, H., Hara, A., Sato, K., Deyashiki, Y., Ninomiya, M., and Sakai, S. (1998) J. Biochem. (Tokyo) 124, 940–946
20. Naramiya, S., Sugimoto, Y., and Ushikubi, F. (1999) Physiol. Rev. 79, 1193–1226
21. Glew, R. H. (1992) in Textbook of Biochemistry with Clinical Correlations (Devlin, T. M., ed) pp. 461–466, Wiley-Liss, Inc., New York
22. Samuelsson, B. (1979) Harvey Lect. 75, 1–49
23. Bydgieman, M., Kwon, S. U., Mukherjee, T., Roth-Brandel, U., and Wiqvist, N. (1970) Acta Obstet. Gynecol. 56, 105–112
24. Oliw, E., Granstrom, B., and Anggard, E. (1983) in Prostaglandins and Related Substances (Pace-Acscia, C., and Granstrom, E., eds) pp. 171–202, Elsevier Science, Amsterdam
25. Mathe, A. A., Hedqvist, P., Strandberg, K., and Leslie, C. A. (1977) N. Engl. J. Med. 296, 910–914
Structural and Mutational Analysis of *Trypanosoma brucei* Prostaglandin H\(_2\) Reductase Provides Insight into the Catalytic Mechanism of Aldo-ketoreductases

Kubata Bruno Kilunga, Tsuyoshi Inoue, Yousuke Okano, Zakaiy Kabututu, Samuel K. Martin, Michael Lazarus, Michael Duszenko, Yuichi Sumii, Yukiko Kusakari, Hiroyoshi Matsumura, Yasushi Kai, Shigeru Sugiyama, Kouji Inaka, Takashi Inui and Yoshihiro Urade

*J. Biol. Chem.* 2005, 280:26371-26382.
doi: 10.1074/jbc.M413884200 originally published online April 21, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413884200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 13 of which can be accessed free at
http://www.jbc.org/content/280/28/26371.full.html#ref-list-1