

Is Renalase a Novel Player in Catecholaminergic Signaling? The Mystery of the Catalytic Activity of an Intriguing New Flavoenzyme

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Running title: Biochemical properties of human renalase

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Abstract

Renalase is a flavoprotein recently discovered in humans, preferentially expressed in the proximal tubules of the kidney and secreted in blood and urine. It is highly conserved in vertebrates, with homologs identified in eukaryotic and prokaryotic organisms. Several genetic, epidemiological, clinical and experimental studies show that renalase plays a role in the modulation of the functions of the cardiovascular system, being particularly active in decreasing the catecholaminergic tone, in lowering blood pressure and in exerting a protective action against myocardial ischemic damage. Deficient renalase synthesis might be the cause of the high occurrence of hypertension and adverse cardiac events in kidney disease patients. Very recently, recombinant human renalase has been structurally and functionally characterized *in vitro*. Results show that it belongs to the *p*-hydroxybenzoate hydroxylase structural family of flavoenzymes, contains non-covalently bound FAD with redox features suggestive of a dehydrogenase activity, and is not a catecholamine-degrading enzyme, either through oxidase or NAD(P)H-dependent monooxygenase reactions. The biochemical data now available will hopefully provide the basis for a systematic and rational quest toward the identification of the reaction catalyzed by renalase and of the molecular mechanism of its physiological action, which in turn are expected to favor the development of novel therapeutic tools for the treatment of kidney and cardiovascular diseases.

Keywords: chronic kidney disease; end-stage renal disease; blood pressure; myocardial ischemia; sympathetic nervous system; catecholamines; oxidoreductase; nicotinamide dinucleotides.

1. Introduction

Renalase was discovered in 2005, through an *in silico* screen of the human genome aimed at identifying genes encoding previously uncharacterized proteins, predicted to be soluble and secreted, which yielded, among others, a gene preferentially expressed in the kidneys [1]. The hypothesis underlying this effort was that kidney endocrine functions possibly included still unknown signaling proteins [2-7]. Over the last six years, an increasing amount of clinical and experimental evidence has been accumulating supporting the idea that renalase is a primary player in the pathogenesis of the cardiovascular events that usually follow renal dysfunctions. While the link between renalase and the pathophysiology of the cardiovascular system seems now to be clear, the molecular mechanism underlying its actions is still obscure in most respects. Several excellent reviews have recently been published on the consequences of renalase deficiency in diabetes, hypertension, cardiac hypertrophy, myocardial ischemia, and stroke [8-13]. This article will mainly focus on the molecular and biochemical properties of mammalian renalase through a critical survey of the often contradictory results published to date on the possible catalytic properties of this protein, with the purpose of discriminating between solid achievements and the many inconsistent observations.

2. The discovery of renalase

Renalase was identified in 2005 by the research team of Gary V. Desir [1], and the story of its discovery is an instructive demonstration of the power of a rationally-designed data mining strategy in the post-genomic era [2,3]. The seminal idea that prompted the search of a still unknown signaling protein released by the kidneys was that traditional pathophysiological mechanisms were insufficient to fully account for the increased risk of cardiovascular adverse events in patients with chronic kidney disease [4,5,14]. Indeed, besides eliminating waste products and maintaining water and electrolyte homeostasis, the kidney also exerts well known endocrine functions (*e.g.* it secretes erythropoietin and calcitriol) and plays a pivotal role in the renin-angiotensin-aldosterone system by releasing the proteinase renin. However, in end stage renal disease, replacement therapy and renal transplant fail to fully restore the functions of the natural organ. Thus, Desir and coworkers concluded that it would be no surprise that “the current endocrine function of kidney was incomplete and that the organ might secrete additional proteins with important biological roles” [1]. To identify them, the Mammalian Gene Collection Project database was screened *in silico* for cDNA encoding proteins predicted to possess these three features: to be uncharacterized, to have a signal peptide for secretion and to lack transmembrane segments. This *a priori* selection yielded 114 hits out of 12,563 distinct open reading frames considered [1]. The candidate genes were then experimentally validated by Northern blot analysis and observing the actual secretion of their products. Just one open reading frame survived these *a posteriori* criteria, showing a robust expression

in the kidney and producing a protein secreted in the medium when transiently expressed in mammalian cultured cells. Finally, the gene product, named renalase by its discoverers, was found in blood plasma and urine of healthy individuals. To date, various research groups have confirmed the presence of renalase in plasma, and solid evidence has accumulated that circulating renalase predominantly originates from the kidneys. However, its discovery has been at least partly serendipitous, since renalase tissue distribution is much wider than initially reported (see Chapter 4), and the peptide originally predicted to represent a secretion signal probably does not serve this role (see Chapter 6).

3. Structure of the renalase gene

The human renalase gene (gene symbol: *RNLS*, formerly *C10orf59*) spans about 300,000 nucleotides from position 90,043,859 to 90,343,082 of the minus strand of chromosome 10 at q23.33. Mapping full-length cDNA sequences to the genome identifies eleven exons, which encode different splicing variants of the protein (Fig. 1). The main isoform (renalase 1, NP_001026879) is composed of 342 residues, with a theoretical molecular mass of 37,847 Da [7,8,15]. The orthologous mouse gene is annotated on chromosome 19C1 [16] and, as detailed in Chapter 7.1, it has been recently inactivated by homologous recombination, providing important experimental clues about its role in the modulation of the cardiovascular system [8,17].

The renalase gene and its main protein product are highly conserved in vertebrates, with amino acid sequence identity above 60% [7,16]. The evident homology between renalase and the vertebrate monoamine oxidase (MAO) A and B genes [1,15] (as well as the intermittent use of the term MAO C to describe the renalase gene product [16]) implicitly suggest a close evolutionary relationship between these three genes. However, neither comparative genomic nor phylogenetic analyses lend support to this hypothesis. The human MAO genes show high levels of overall colinearity and similarity to a family of prokaryotic MAOs (typified by the *aofH* flavin-containing MAO gene product of *Mycobacterium tuberculosis* H37Rv over 85% of its length). Renalase however, shares extensive colinearity with members of a distinct and widely distributed FAD dependent oxidoreductase family (*e.g.* 27% identity and 46% similarity with *Cyanothece* sp. ADN1670 over 95% of its length). Indeed, the human MAO A and renalase protein sequences share only 38% identity in the 10% of the renalase sequence that aligns well with the human MAO A polypeptide. These observations immediately suggest that, rather than deriving from a eukaryotic specific gene duplication, the ancestor of the MAO genes on one hand and renalase on the other were independently acquired by eukaryotes from prokaryotic forbearers.

Unfortunately, contemporaneous, cross kingdom phylogenetic analyses of MAO-like and renalase-like genes are impossible due to low numbers of unambiguously aligned residues. However, MAO-like genes are found in mycetozoa, many fungi, plants and some protists. Among non-metazoan eukaryotes, gene products displaying overall

similarity with renalase are observed in plants and some stramenopiles but not in fungi (although the N-terminal region of renalase shows significant similarity to portions of fungal prenylcysteine lyase proteins). Phylogenetic reconstructions of selected MAO-like protein sequences lend moderate support to the monophyly of eukaryotic genes, but fail to identify a well-supported prokaryotic sister group, suggesting that such genes have been present within eukaryotes since their origin, but not affording insight into the nature of their prokaryotic donor. The evolutionary history of eukaryotic renalase genes is even less clear, with animal, plant and oomycete genes potentially deriving from independent acquisitions from prokaryotes. In any case, these considerations indicate that inferences regarding the role of renalase should be derived from studies of MAOs only with great caution as these amine-oxidase-like subfamilies are only distantly related to one another (not shown).

4. Renalase isoforms and gene expression pattern

Different mRNA transcripts of *RNLS* have been detected and sequenced, highlighting the existence of protein variants originating from alternative splicing [8,18,19]. Besides the aforementioned renalase 1 (NP_001026879), a second annotated protein isoform exists (renalase 2, NP_060833), with a slightly shorter polypeptide chain (315 residues) and a different sequence in its C-terminal region. The other two characterized *RNLS* transcripts (AK296262 and BX648154) encode much shorter deduced polypeptides (233 and 138, respectively). The comparison of the primary structures of the alternatively spliced renalase isoforms in the light of the crystal structure of renalase 1 [20] suggests that, while renalase 2 would probably be a compact globular protein similar to its larger isoform, the other two polypeptides would be unlikely to yield flavin-containing proteins, since they lack essential structural elements for FAD binding (see Chapter 6). Thus, the potential physiological significance of such shortest variants is uncertain. In the case of the mouse orthologous gene, two transcript isoforms are annotated: the first (NM_001146342) has intron-exon structure identical to human NP_001026879 and its translation product has been partly characterized [16]; the second lacks exons 2 and 3 (as does human transcript AK296262) (see Fig. 1), but encodes a protein whose deduced C-terminal region is identical to that of NP_001026879. This suggests that isoform sampling in human tissues is still incomplete, and further alternative transcripts might exist.

In the most comprehensive study on the pattern of *RNLS* expression published so far, autoptic human tissues samples known to express MAOs have been analyzed both by immunoblotting using an anti-renalase monoclonal antibody and by reverse transcriptase polymerase chain reaction [19]. In addition to kidney and myocardium [1], renalase was found in forearm vein and artery, renal vein and artery, ureter, median nerve, hypothalamus, pons, medulla oblongata, cerebellum, pituitary gland, cortex, and spinal cord [19]. In the kidneys, renalase was shown to be present in glomeruli and proximal tubules [1]. Widespread transcription of the human renalase gene is also confirmed by

microarray data [21] and Whole Transcriptome Next-Generation Sequencing (RNA-Seq) [22]. Renalase 1 is the only isoform apparently detected in blood plasma and urine and it represents the major isoform in all tissues tested (kidney, heart, skeletal muscle, liver, testicle, hypothalamus, adrenal gland) [1,19]. Transcripts encoding renalase 2 and the two shortest variants (AK296262 and BX648154), although at lower levels than renalase 1 mRNA, were observed in all samples examined, while no renalase 2 transcript was detected in the hypothalamus [19]. A renalase concentration of 0.1 mg per gram wet tissue has been estimated in the kidneys by immunoblotting [10]. Data on the absolute concentration of renalase in human blood plasma have been explicitly reported only recently, as determined by ELISA, showing that it is about 4 $\mu\text{g/ml}$ in healthy individuals, corresponding to 0.1 μM [23-28]. Murine renalase gene expression was observed by reverse transcriptase PCR in kidney, testicle, liver, heart, 12.5-days whole embryo, brain, and skeletal muscle [16].

Several hundred single nucleotide polymorphisms (SNPs) of *RNLS* are known, most of which are located in the flanking regions, within the introns or the untranslated regions of the gene and thus do not result in missense mutations, although they could possibly affect gene expression and mRNA splicing. The twenty-four SNPs resulting in amino acid replacements are shown in Table 1. Interestingly, most of the mutated residues are located on the molecular surface of renalase 1, at sites where the replacements are expected to have little impact on protein conformational stability. Several of the SNPs mapping in the interior of the renalase molecule maintain the hydrophobic character of the residue side chain, with the exceptions of Ile111Thr, Ala195Ser, Ile226Thr and Pro240Ser. The only allelic variant involving a residue interacting with FAD carries the Met161Ile replacement, in which the substitution of the side chain, which stacks on the FAD adenine ring, does not seem to put at risk the binding of the cofactor. As reported in Chapter 7.3, for a few *RNLS* SNPs (listed in Table 2) the possible association of a specific allele or genotype to some pathological conditions has been studied (see Chapter 7.3).

Bioinformatics analysis, physiological and clinical evidence, and experimental data indicate that renalase is at least partially secreted. First of all, as mentioned above, renalase 1 was detected in body fluids such as blood plasma and urine [1,10,29,30]. Secondly, in patients suffering from chronic kidney disease and primary glomerulonephritis, as well as in animal model of kidney failure, extracellular renalase was absent or present at lower concentrations, indicating the kidneys as the main source of the secreted protein [1,30-32]. Finally, mammalian cells transfected with constructs expressing either human or mouse renalase were shown to secrete the protein into the culture medium [1,16]. However, as discussed in Chapter 6, it is unlikely that the N-terminal region of renalase, initially proposed as a secretion signal [1], could be processed by the conventional cell secretory pathway, because the cleavage of this peptide would dramatically destabilize the native conformation of the protein.

5. Biochemical properties of renalase

5.1. Purification of endogenous and recombinant renalase forms

Any proposal about the mechanism of the physiopathological action of a newly discovered protein needs to be verified in the context of its functional and structural properties. In the case of renalase, the application of this general rule had to wait several years until sufficient amounts of stable recombinant holoprotein became available for biochemical characterization, which eventually led also to the characterization of its crystal structure. The only attempt to characterize endogenous human renalase was carried out on the protein isolated from the urine of healthy volunteers [1]. Excreted renalase was purified by ammonium sulfate precipitation followed by immunoaffinity chromatography using antibodies against the recombinant protein. Unfortunately, essentially no biochemical characterization was performed on the purified material, except for electrophoretic analysis and catalytic activity assays (see Chapter 5.2). Thus, although SDS-PAGE provided a molecular mass (35,000 Da) slightly lower than that predicted for the full length 342-residue polypeptide (37,847 Da), the accuracy of the estimate did not allow definitive conclusions about the presence or absence of the signal peptide after post-translational processing of urine renalase. Even more surprisingly, the possible presence of a bound flavin cofactor was not verified in the purified protein [1].

The production of recombinant mammalian renalase in different hosts using various expression strategies has been described by four independent groups. Desir's group reported the production of two recombinant forms of human renalase in *Escherichia coli*. Initially, the protein was expressed as an N-terminal fusion with glutathione S-transferase (GST) and purified in soluble form by affinity chromatography on a Glutathione Sepharose column [1,17]. GST-renalase was used to raise anti-renalase polyclonal antibodies and to study its catalytic activity *in vitro*. Later, two recombinant allelic isoforms of human renalase were synthesized with no tag or fusion partners, extracted from *E. coli* inclusion bodies by chaotropic agents and refolded *in vitro* in the presence of FAD [17,33]. Wang and coworkers produced human renalase in *E. coli* as a fusion protein containing at the N-terminus the *pelB* leader sequence for localization in the cell periplasm, and a C-terminal His-tag. The abundant 38 kDa product was purified (although it was not specified whether under denaturing or non-denaturing conditions) and used for monoclonal antibody production [34]. Zhang's group reported the synthesis of *Mus musculus* renalase in *E. coli* as an N-terminal fusion with GST, with no purification attempt [16].

Recombinant renalase has also been produced in eukaryotic cells, although its isolation from this source has never been reported. Mouse renalase has been successfully synthesized in human embryonic kidney cells as a C-terminally enhanced green fluorescent protein (EGFP)-tagged fusion [16]. The expression of human renalase in insect cells by a baculovirus-based system was described, although many details of the cloning procedure are missing and no

explanation is given of the very large apparent molecular mass of the expression product (85 kDa) [35]. The same authors also reported the synthesis of human renalase in embryonic kidney cells [36]. Finally, the production of human renalase in the yeast *Pichia pastoris* has been described in a patent by Desir and coworkers [37]. Gene expression was obtained using the pPICZ α (Invitrogen) vector, which promotes its integration in the host genome and secretion of the resulting translation product in the growth medium, where it was detected immunochemically.

Despite the ability of renalase to incorporate a flavin nucleotide being an absolute prerequisite for its initially proposed enzymatic action, the actual presence of FAD or FMN in the mentioned recombinant renalase forms was not reported. Since the presence of flavin nucleotides is difficult to miss due to its intense yellow color, we suspect that no purification procedure yielded a stable flavoprotein. Using the pGEX-4T-2/mMAO-C plasmid kindly provided by Dr. Shu-Ping Zhang [16], we obtained the synthesis in *E. coli* of limited amounts of soluble mouse GST-renalase, which, after successful isolation by affinity chromatography, contained no flavin cofactor (Aliverti, unpublished results). Apparently, the only observation (reported in its first article on this subject) that led Desir to conclude that human renalase is a flavoprotein was that inclusion of 0.1 μ M FAD in the bacterial culturing medium was required in order to isolate a recombinant protein that demonstrated oxidoreductase active [1]. This is an inconsistent observation, since it has long been known that FMN or FAD biosynthesis by *E. coli* is not a limiting factor in flavoprotein production, even when expression levels exceed 25 mg of target protein per gram of bacterial cells [38,39]. Indeed, Medvedev and coauthors clearly stated that a conclusive proof that renalase contains FAD was still lacking [9]. This proof was obtained in the same year, when after several expression trials in both *E. coli* [40] and *Saccharomyces cerevisiae* [Aliverti, unpublished results], we finally obtained limited amounts of highly purified human flavin-containing renalase spontaneously folded *in vivo* in the bacterial host [40]. Both Glu37 and Asp37 allelic isoforms of renalase were found to contain 1 mol FAD per mol protein, which, at variance with MAO enzymes, was tightly but not covalently bound to the apoprotein. The fluorescence of the bound cofactor was found completely quenched, and circular dichroism spectrophotometry indicated that its isoalloxazine ring is embedded in a highly asymmetrical environment, markedly different from that of MAOs [40]. Dynamic light scattering, far ultraviolet circular dichroism, sulfhydryl titration and mass spectrometry predicted a globular, highly packed conformation in solution, with no disulfide bonds and an α and β secondary structure content of 23% and 21-25%, respectively [40]. By thermal denaturation at low ionic strength and neutral pH, an apparent melting temperature of 54 °C was determined for recombinant renalase, indicating that its native conformation is particularly stable [Aliverti, unpublished results]. Under non-denaturing conditions, renalase was also very resistant to the action of a variety of proteases, indicating the absence of large flexible surface loops. The protein was digested only in the presence of urea at concentrations above 1.5 M, without detectable transient intermediate polypeptides, indicating a global native conformation stabilized by highly cooperative tertiary contacts

[Aliverti, unpublished results]. Production of renalase in *E. coli* with either an N-terminal His-tag, C-terminal His-tag, or a N-terminal cleavable SUMO polypeptide did not affect its biochemical properties, suggesting that the recombinant protein was not altered in any way by the fusion strategy [40, Aliverti, unpublished results]. All the above indirect conclusions about the structural properties of renalase are in full agreement with the crystal structure of the protein (see Chapter 6).

5.2. Functional properties of renalase

Only two groups have published data on the biochemical *in vitro* properties of renalase to date. In their seminal 2005's paper, Desir and coworkers reported that both recombinant GST-renalase fusion and the natural protein isolated from urine possessed amine oxidase activity with a substrate preference profile different from those of either MAO A or MAO B [1]. Dopamine was described as the best renalase substrate, followed by epinephrine and then norepinephrine, whereas its activity towards other biogenic amines was negligible. Moreover, renalase activity was unaffected by clorgyline and pargyline, specific inhibitors of MAO A and B, respectively [1]. Based on this observations, mammalian renalase was proposed to represent a new type of MAO [1,7,15], which was later named MAO C [16], a term under which is currently classified in some databases. These conclusions were strongly questioned by Frans Boomsma and Keith F. Tipton [41], who pointed out that copper enzyme semicarbazide-sensitive amine oxidase is the only enzyme responsible for the degradation of catecholamines in mammalian blood plasma. The supposed activity of purified renalase, measured only as H₂O₂ production, might be due to catecholamine outoxidation, which is known to be relevant at pH above 7 in the absence of antioxidants. Alternatively, since the reaction was followed for just 0.25% of the expected total amine conversion, this activity might be due to the oxidation of contaminant(s) that are common in commercial catecholamines. Finally, they argued that, even if renalase were actually able to degrade biogenic amines, its reported turnover number is so low ($> 0.25 \text{ min}^{-1}$) [1], that it would hardly affect blood catecholamine concentration [41].

Subsequently, Desir's group proposed a catecholamine-triggered activation mechanism for circulating renalase, which would exist in inactive form under normal condition [29]. These authors reported that, while amine oxidase activity is undetectable in rat plasma under basal conditions, a 2 min infusion with epinephrine or dopamine determined the rapid (<1 min) onset of renalase activity (assayed as H₂O₂ production specifically inhibited by an anti-renalase antibody) that lasted for several minutes. Since the plasma concentration of renalase did not follow the same time course, increasing only ca. 10 min after perfusion, they concluded that the rapid rise of amine oxidation activity was due to the conversion of an inactive prorenalase form to the functional enzyme. Consistent with this finding, they also observed that urine human renalase displays a much higher turnover rate than the plasma form. Since blood plasma

was found to have a strong inhibitory effect on purified urine renalase, they concluded that circulating prorenalase might represent a resting enzyme form, possibly complexed with a specific inhibitor [8,18,29].

As reported in Chapter 5.1, recently we obtained a recombinant *in vivo*-folded FAD-containing human renalase. At variance with previous reports, we found that our protein, although eliciting the expected effects on blood pressure when injected into rats, was completely devoid of any amine oxidase activity [40]. At the same time, Desir and coauthors reasoned that, since the renalase sequence contains the dinucleotide binding motif GXGXXG, which is the hallmark of the Rossmann fold, it could bind either NAD or NADP [17,33,42]. Indeed, they found that *in vitro* refolded recombinant human renalase possesses NADH-oxidase activity, with a K_m^{NADH} of $15 \pm 1 \mu\text{M}$, and a V_{max} of $15 \pm 0.1 \text{ nmol/min/mg}$, corresponding to a k_{cat} of 0.40 min^{-1} [17]. The enzyme was inactive towards NADPH. When epinephrine was included in the reaction mixture, it was degraded at a rate 18-fold faster than in the absence of NADH through a reaction that was abolished by superoxide dismutase but not by catalase. NADH-dependent epinephrine degradation by renalase displayed a $K_m^{\text{epinephrine}}$ of $17 \pm 4 \mu\text{M}$, and a V_{max} of $22 \pm 0.1 \text{ nmol/min/mg}$, corresponding to a k_{cat} of 0.58 min^{-1} [42]. On these bases, the authors stated that renalase is a new type of FAD-containing, NADH-dependent, catecholamine degrading enzyme, although no explanation was given of how the same Rossmann fold domain could bind both FAD and NADH.

Serious doubts over the putative amine-degrading activity of renalase were again raised by Nina Eikelis and coauthors [43], who pointed out that: (i) the supposed renalase catecholamine-degrading rate was too low to be ascribed to a real enzyme activity, (ii) a structurally sound recombinant renalase produced by others did not metabolize catecholamines [40], and (iii) renalase gene inactivation was studied in a mouse strain (C57BL/6 [17]) that synthesized a shortened form of renalase, which lacks the FAD/NAD-binding motif, and thus expected to be enzymatically inactive. Desir replied that the renalase knock out was maintained in a mixed mouse background (129Sv/J and C5BL/6), where the former wild-type strain produces a full-length renalase, and the reason for the discrepancy between enzyme activity data obtained in different laboratories depends on the fact that Aliverti's group omitted NADH in the assays [43].

Another intriguing aspect of renalase function is the role played by the residue 37 side-chain in catalysis. As mentioned in Chapter 4, the *RNLS* rs2296545 G allele, encoding the Asp37 variant of renalase, was found to be associated with cardiovascular pathologies [33,44,45]. The catalytic properties of both Asp37 and Glu37 renalase isoforms were compared by studying their NADH-diaphorase activity, measured with the water-soluble salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) as an artificial electron acceptor. The Glu37Asp replacement determined an increase in K_m^{NADH} from 34 ± 4 to $820 \pm 115 \mu\text{M}$, and a decrease in V_{max} from 58 ± 1 to $25 \pm 2 \text{ nmol/min/mg}$ [33]. The effect of the conservative Glu37Asp mutation was interpreted on the basis of the critical role played by the corresponding residue in MAOs (Glu34 in MAO B) and other flavoenzymes sharing the same

FAD-binding motif [17]. In such enzymes the Glu γ -carboxylate interacts with the adenosyl ribose moiety of bound FAD, controlling their catalytic properties, as demonstrated by the large impact of the Glu34Asp mutation on activity [46,47].

Very recently, we compared the functional properties of the Glu37 and Asp37 isoforms of recombinant *in vivo* folded renalase [20, Aliverti, unpublished results] and found no difference between them, in sharp contrast with the aforementioned report [33]. Moreover, we provided a solid confirmation that renalase slowly reacts with both NADH and NADPH, and weakly binds the oxidized forms of both dinucleotides. We found that renalase catalyzes NADH- and NADPH-dependent diaphorase reactions with various artificial electron acceptors (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT) being the preferred one), and determined the following steady-state kinetic parameters: $k_{\text{cat}}^{\text{NADH}} = 0.14 \text{ min}^{-1}$, $k_{\text{cat}}^{\text{NADPH}} = 0.26 \text{ min}^{-1}$, $K_{\text{m}}^{\text{NADH}} = 18 \text{ }\mu\text{M}$, $K_{\text{m}}^{\text{NADPH}} = 175 \text{ }\mu\text{M}$ [20]. In addition to indicating that Glu37 has no role in the catalysis of these reactions, our steady-state kinetic data markedly differ from those published by Desir's group in showing that renalase is not strictly specific for NADH [20]. Moreover, we demonstrated that protein-bound FAD is involved in these reactions, showing its reduction at rates compatible with catalysis, when renalase is incubated under anaerobiosis with either NADH or NADPH. By differential spectrophotometry we determined that the dissociation constants of the protein for NAD^+ , NADP^+ and 2-phospho-AMP were all in the range of 1-2 mM. Taken together, the very low turnover numbers, the low affinity for nucleotides, and the poor selectivity in discriminating among them, strongly suggest that renalase is not a NAD(P)H-dependent enzyme, and that the observed diaphorase activity indicates a physiologically irrelevant side reaction [20]. Finally, we showed that renalase slightly stabilizes the neutral form of FAD and that is able to form a sulfite adduct [20]. These observations indicate that the reactivity of the FAD prosthetic group of renalase dramatically differs from that of MAO A and B, allowing us to conclude, in agreement with the phylogenetic data (see Chapter 3), that renalase is not a MAO-like enzyme and likely is not even an oxidase.

6. Three-dimensional structure of renalase

The crystal structure of human renalase isoform 1 (PDB ID 3QJ4) was solved at 2.5 Å resolution by molecular replacement using a putative oxidoreductase from *Pseudomonas syringae* q888a4 (PDB ID 3KKJ) as starting model [20]. The renalase molecule has a compact, elongated, globular shape, with α and β secondary structure content of 26% and 25%, respectively. It is organized in two domains: one consisting of three non-adjacent polypeptide stretches, and the other by the two intervening segments. At the interface between the two domains, a wide and deep cleft runs perpendicularly to the longer axis of the molecule on the side opposite to the entrance of the active-site cavity described

below. The FAD cofactor is buried within the interior of the molecule, with the exception of a few small regions, including part of the isoalloxazine ring, and is firmly, but not covalently bound to the protein through several H-bonds and other contacts. The first domain, as predicted by the presence of the dinucleotide binding motif, adopts the classical Rossman fold, which is used to bind the FAD prosthetic group [48], thus excluding the possibility that it could provide a NADH-binding site, as proposed by other authors [33]. The second domain, based mainly on an antiparallel five-stranded beta sheet surrounded by three helices and a β hairpin, is presumably involved in substrate binding. The overall fold unequivocally classifies renalase as a member of the *p*-hydroxybenzoate hydroxylase (PHBH) protein superfamily [49], which comprises several flavoenzymes catalyzing highly diverse reactions (Table 3). The oxidases of this superfamily belong either to the L-amino acid oxidase or to the D-amino acid oxidase structural families, renalase more closely resembling the former enzymes, which encompass also MAOs. In comparison to most of its structural homologs, renalase lacks a third domain, which in PHBH is named the interface domain [50], and which participates in substrate binding in MAOs and polyamine oxidase [51]. Due to the absence of this structural element, the polar, positively charged cavity of 224 \AA^3 that faces the *re* side of the flavin ring and presumably represents the active site is freely accessible to the solvent through a large opening [20].

As renalase was claimed to degrade catecholamines by either oxidase or NADH-dependent reactions [1,33], these proposed catalytic activities have been considered in the light of its three-dimensional structure [20]. As shown in Fig. 2, the renalase active-site markedly differs from those of MAOs and related amine oxidases: the former lacks both the 'aromatic cage' that in the latter enzymes binds the substrate amine group and promotes its oxidation, and a lysine (Lys 305 and Lys296 in MAO A and MAO B, respectively) conserved in most of the oxidases of the superfamily [20]. These features refute the classification of renalase as a MAO, suggesting that probably it is not an oxidase at all. In principle, the observed NADH reactivity of renalase [33] is suggestive of a possible monooxygenase (*i.e.* hydroxylase) activity, substantiated by its structural similarity to PHBH, the prototype of flavin-dependent aromatic hydroxylases [52]. Such activity is compatible with the observed very slow reaction between renalase and NADH or NADPH [20], since in aromatic hydroxylases the reductive half-reaction (FAD reduction) is exceedingly slow in the absence of a hydroxylatable substrate [52]. Moreover, the lack of a typical NAD-binding site in renalase [20] is in line with this hypothesis, because PHBH binds NADPH at a site [53] that corresponds to the aforementioned interdomain cleft of renalase, which could play an equivalent role. However, control of FAD reduction and reactivity of the flavin-C4a-hydroperoxide intermediate is obtained in aromatic hydroxylases through large conformational transitions occurring during the catalytic cycle, in which the isoalloxazine ring oscillates, alternating between 'in' and 'out' conformations [52]. When the flavin adopts the 'in' conformation, its N5 atom is strictly protected from the solvent to avoid H_2O_2 release from the flavin peroxide, a reaction competing with substrate hydroxylation [52]. As reported elsewhere [20],

the swing of the flavin ring to the 'out' position is precluded in renalase by the obstructing presence of the β 18 strand and particularly by the Trp288 side-chain (Fig. 2). Notably, in renalase the isoalloxazine N5 position is solvent exposed [20]. In addition to the Rossmann fold GXGXXG signature, flavoprotein hydroxylases display two highly conserved consensus sequences: the 'GD' and the 'DG' motifs [54]. Whereas, the former polypeptide region participates in FAD binding and is conserved in renalase, the latter motif, critical for the interaction with the nicotinamide nucleotide, is absent. In particular, Gly160 and His162 of PHBH, which are very important for NADPH binding [54], in renalase are both replaced by Pro residues (Pro162 and Pro164), excluding the presence of a functional NAD(P)-binding site in this protein. These considerations, combined with the observation that the FAD structural environment is incompatible with the required chemistry (confirmed by the production of superoxide upon reaction with O_2 [33] instead of hydrogen peroxide as typical in hydroxylases [52]), led to the logical conclusion that renalase cannot be a monooxygenase. We proposed that the slow NAD(P)H-dependent activity of renalase is a side reaction arising from non-physiological access of the nucleotides to the flavin ring, driven by the positive charge of the wide active-site cavity [20].

As mentioned in Chapter 5.2, Glu37 was proposed to modulate the NADH-dependent activity of renalase by interacting with the FAD ribose moiety [17]. Structural data ruled out this hypothesis, by showing that Glu37 is part of a loop near the rim of the surface cleft [20], in agreement with our observation that the Glu37Asp replacement had no effect on the properties of the flavoprotein [20].

As reported in Chapter 4, a plausible N-terminal signal peptide for secretion (of 16-17 residues) was predicted in renalase [1], although SignalP 4.0 [55] assigns it just a borderline score. Inspection of the protein crystal structure shows that this region corresponds to the central β 1 stand and half of the adjacent α 1 helix that are integral part of the Rossmann fold [20] and whose removal would cause the collapse of the entire FAD-binding domain, as shown in Fig. 3A. This consideration tends to exclude that the 1-16 peptide represents a signal for secretion, or that it is cleaved during protein processing. To reconcile this inference with the observation that renalase is actually present in blood plasma, a non-conventional secretion mechanism must be invoked [56].

Finally, the availability of the three-dimensional structure of the isoform 1 (NP_001026879) of renalase allows the development of "educated guesses" about the possible structural features of the alternative splicing isoforms. Renalase 2 (NP_060833) originates from alternative splicing between exons 6 and 7, causing a frame shift that alters the C-terminal sequence and makes the polypeptide chains 27 residue shorter through the introduction of a premature stop codon (Fig. 1) [19]. This profoundly modifies the sequence of the region corresponding to the β hairpin β 19- β 20 and precisely deletes the two C-terminal helices η 2 and α 11 of isoform 1 [20], as outlined in Fig. 3B. These structural modifications are expected to make FAD more exposed in renalase 2 than in renalase 1 and to significantly affect the features of its active-site cavity. In comparison to renalase 1, the splicing isoform AK296262 lacks the polypeptide

segments encoded by exons 2 and 3 (Fig. 1). As a result, the surface exposed $\alpha 4$ helix of the FAD-binding domain and the $\beta 3$ - $\beta 4$ hairpin and the $\beta 5$ - $\alpha 2$ - $\alpha 3$ - $\beta 6$ - $\beta 7$ subregion of the substrate-binding domain are deleted [20]. The resulting protein would lack critical residues for the binding of the FAD isoalloxazine, suggesting that it would not be able to incorporate the cofactor and hence to be active as an oxidoreductase. In the variant BX648154 the FAD-binding domain is almost completely deleted, resulting in a polypeptide with unpredictable properties.

7. Physiological roles of renalase and their impairment as possible pathogenic mechanisms of cardiovascular diseases

Despite our appreciation of the probable molecular mechanism of its physiological activity, the nature of the catalyzed reaction, and the identity of its substrate(s) is very poor (see Chapter 5), there is quite solid and constantly accumulating evidence of the important roles played by renalase in the control of blood pressure and heart function. Since its discovery, two key observations were made about renalase pathophysiological actions: 1) its concentration in blood plasma is dramatically lowered in subjects suffering from severe kidney disease; and 2) the parenteral administration of the recombinant protein has a hypotensive effect in rats [1]. For clarity, the proofs of the relationship between renalase and cardiovascular system pathophysiology will be subdivided here in experimental, clinical and epidemiological aspects.

7.1 Experimental evidence of renalase action on the cardiovascular and sympathetic systems.

The finding by Desir's group that intravenous injection of recombinant renalase in healthy rats decreases, in dose-dependent manner, systolic, diastolic, and mean arterial pressure, as well as heart rate and contractility [1] has been partially confirmed by us [40]. In addition, it was later shown that subcutaneous administration of the protein has a profound effect on blood pressure and heart rate in an animal model of hypertension (Dahl salt-sensitive and 5/6 nephrectomized rats; [10,57]). Very interestingly, using an isolated heart model of acute coronary syndrome, perfusion with recombinant renalase was shown to exert a strong protective effect against ischemia, preserving ventricular function and reducing myocardial necrosis and infarct size [58]. A major breakthrough in the identification of renalase physiological role has been the inactivation of the *RNLS* gene in mouse by homologous recombination [17]. In comparison to littermates, KO mice displayed 25% lower body mass, tachycardia, hypertension, and higher circulating norepinephrine levels, while markers of endothelial function were unaffected. Moreover, gene inactivation was found to greatly increase the extent of myocardial damage following induced ischemia on isolated heart, an effect that was fully prevented by renalase infusion [17]. Since *RNLS* KO was also shown to be associated to a markedly decreased

NAD⁺/NADH ratio in the myocardium, it was proposed that the cardioprotective effect of renalase might be mediated by the control of both sympathetic activity and redox potential of the NAD⁺/NADH couple, which in turn is expected to affect energy metabolism and sirtuin-1 signaling [17]. However, the exceedingly low turnover of renalase measured in NADH-dependent reactions *in vitro* (see Chapter 5.2) would exclude direct effects on NADH and/or NAD⁺ concentrations *in vivo*. Very recently, using an animal model of chronic kidney disease, *i.e.* 5/6 nephrectomized rats, Baraka and El Ghotny showed that prolonged renalase administration after the surgical procedure significantly controlled the plasma noradrenaline rise induced by nephrectomy, ameliorating the resulting cardiovascular complications, including hypertension, myocardial fibrosis and cardiac hypertrophy [59].

A link between renalase and catecholamine metabolism emerges from several studies. Downregulation of *RNLS* expression in healthy mice by antisense phosphorothioate oligonucleotides was found to produce both a rise in blood pressure and an increased sensitivity to norepinephrine injection [8,60]. In various rat models of chronic kidney disease, lower concentrations of renalase in kidneys, heart and blood were always accompanied by increased levels of epinephrine and norepinephrine in plasma and heart [30,32,61,62]. Furthermore, some of these studies together with other investigations on rodent models showed that renalase levels inversely correlate with the concentration of dopamine, which has hypotensive and cardioprotective actions at variance with other catecholamines [63], in kidneys and urine [32,64]. The known natriuretic action of dopamine and its recently proposed participation in the modulation of phosphate tubular reabsorption have also suggested a role for renalase in the control of sodium and phosphate ions homeostasis [64].

7.2 Clinical evidence for renalase actions on the cardiovascular and excretory systems.

The involvement of renalase in the renal dopaminergic system was substantiated by a clinical report on eight kidney transplant recipients, showing a correlation between the increase in dopamine concentration and the decline of renalase excretion in urine [65]. Other reports highlighted the involvement of renalase in different forms of hypertension. First, in a study on neurogenic hypertensive subjects, where noradrenaline spillover from adrenergic nerves was significantly increased, renalase secretion by the kidneys was undetectable in most patients [66]. Furthermore, biopsies from twenty-three patients affected by IgA nephropathy, the most common form of primary glomerulonephritis, displayed decreased renalase levels in tubular epithelial cells, correlating with both pathology chronicity indexes and hypertension [31]. Recent studies on the impact of kidney and heart transplantation on the level of circulating renalase led to the unexpected finding that plasma renalase concentration increases after organ implantation and the degree of increase correlates with the severity of kidney failure in the allograft recipients [23-25]. The same authors also investigated in chronic kidney disease patients the impact of hemodialysis and peritoneal dialysis

on blood serum renalase They concluded that impairment of renal function correlates with increased renalase levels [26-28], which was found up to 10-fold higher in anuric patients that underwent bilater nephrectomy in comparison to healthy subjects [26]. Finally, it was reported that stroke and hypertension were associated to lower serum renalase concentrations in a hemodialysis population [27].

7.3 Epidemiologic data implying renalase gene polymorphisms as disease risk factors.

To date, four independent genetic studies have highlighted a correlation between four individual *RNLS* SNPs and the propensity to develop specific pathological conditions in different populations, as summarized in Table 2. In the first such study, carried out on 1317 hypertensive and 1269 normotensive subjects recruited from the International Collaborative Study of Cardiovascular Disease in Asia (InterASIA), *RNLS* was shown to represent a susceptibility gene for essential hypertension in the northern Han Chinese population [44]. In particular, the G and the C alleles of the rs2576178 and rs2296545 SNPs, respectively, displayed significant higher frequencies in the pathological group. Genotyping suggested a codominant model for the expression of both risk-associated alleles [44]. The association of rs2576178, which maps in the 5' flanking region of the renalase transcript, with hypertension was confirmed by a case-control study on 400 Caucasian end-stage renal disease patients of Polish origin under dialysis [67]. The same survey also found a significant correlation of the G allele of the rs10887800, mapping in intron 6, with increased incidence of hypertension. A similar study, carried out on 900 type 2 diabetic patients and control individuals from a population of Polish origin found a significant correlation between the rs2576178 G allele and type 2 diabetes, and a strong association of the rs10887800 G allele with stroke in hypertensive individuals, regardless of their diabetic status [45]. Interestingly, *RNLS* was recognized as a novel gene responsible for type-2 diabetes by a genome wide association scan in the Amish population, which identified rs2437871 as a disease-linked SNP [68]. Among the *RNLS* SNPs considered in the above studies, rs2296545 is the only resulting in variants of the encoded protein, which differs for the presence of a Glu (G allele) or an Asp (C allele) residue at position 37. The homozygous CC genotype was recently shown to be associated with cardiac hypertrophy, ventricular dysfunction, poor exercise capacity and higher susceptibility to induced ischemia [33], through genotyping 590 Caucasian individuals from the Heart and Soul Study [69],

8. Concluding remarks

As foreseen by Eberhard Ritz, who, soon after the discovery of renalase, wrote that “it is easy to predict that in the near future this novel endocrine product of the kidney will be intensely investigated experimentally and in renal patients” [2], this protein has been the subject of several studies that, over the last seven years, have considerably

strengthened its connection with the physiopathology of the cardiovascular and excretory systems. Moreover, the involvement of renalase in the modulation of the catecholaminergic system has become increasingly clear. Thus, human renalase is now regarded as a new player in the control of blood pressure and heart function, whose modulation could lead to promising new pharmacological treatments of various cardiovascular dysfunctions [3,8,13,23,24,70,71]. Renalase has been proposed as a drug for replacement therapy in end stage renal disease [12,17,59,70,72], as early biomarker of acute kidney ischemia [10] and essential hypertension [8], as well as prognostic factor for stroke [27,45], and even as possible target for the therapy of psychiatric disorders caused by altered catecholaminergic signaling in the central nervous system [19]. Obviously, development of effective tools for pharmacological intervention requires the mechanism of renalase action to be known at the molecular level. Unfortunately, this is not the case yet, since the supposed catalytic activity of renalase as a catecholamine-degrading enzyme has recently been proved wrong by the detailed biochemical characterization of the recombinant human protein [20,40]. However, the structural and functional data on renalase should provide the basis for a systematic and rational quest toward the identification of its substrates and the catalyzed reaction.

List of abbreviations

MAO, monoamine oxidase; GST, glutathione S-transferase; SNP, single nucleotide polymorphism; PHBH, *p*-hydroxybenzoate hydroxylase.

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Figure legends

Figure 1. Schematic representation of the structure of the human renalase gene and its major expression

products. The schematic intron/exon organization of the known splicing variants of the *RNLS* transcripts is shown. On the right, the deduced amino acid sequence of the *RNLS* exons is reported.

Figure 2. Comparison between the active site of renalase and those of MAO A and MAO B.

Stereo view of the putative active site of human renalase overlaid on those of MAO A (**panel A**) and MAO B (**panel B**). The FAD isoalloxazine ring and relevant amino acyl residues of renalase (PDB ID 3QJ4, chain A) are shown in blue (black in the printed version of the article), whereas the corresponding residues of MAO A (PDB ID 2BXR, chain A) and MAO B (PDB ID 1GOS, chain A), including the ‘aromatic cage’ (Tyr407 and Tyr444 in MAO A, and Tyr398 and Tyr435 in MAO B) are depicted in gold and pink, respectively (both white in the printed version of the article).

Figure 3. Putative renalase signal peptide and structural differences between renalase 1 and 2.

Ribbon model of the three-dimensional structure of human renalase (PDB ID 3QJ4, chain A). **Panel A**, Crystal structure of renalase 1, showing the 1–16 N-terminal peptide, proposed to represent a secretion signal, in orange (light grey in the printed version of the article). **Panel B**, Hypothetical model of renalase 2 (NP_060833), in which the shorter C-terminal peptide encoded by exon 7c replaces the longer one encoded by exon 7b (see Fig. 1). Renalase 2 is predicted to lack the α 11 helix of renalase 1 and to present a different amino acid sequence in the region highlighted in red (grey in the printed version of the article), which includes β 19 and β 20 strands.

Table 1. Known single nucleotide polymorphisms of the renalase gene that result in protein amino acyl substitutions.

SNP accession no.	Experimental evidence ^a	Exon no. ^a	Base change	Amino acyl replacement
rs2296545	genomic	1a	C/G	Asp37/Glu37
rs147588689	cDNA	2	A/G	Arg75/Cys75
rs143513862	cDNA	3	C/T	Val85/Ile85
rs79981780	cDNA	3	C/T	Ile93/Val93
rs149825485	cDNA	3	C/G	Asp102/Glu102
rs140158928	cDNA	3	A/G	Ile111/Thr111
rs139294588	genomic	4	C/G	Gln134/His134
rs146646870	cDNA	4	C/T	Glu143/Lys143
rs77594193	genomic	4	A/G	Pro151/Leu151
rs41317260	genomic	4	A/C	Met161/Ile161
rs78525460	cDNA	5	G/T	Gln181/Lys181
rs112858030	genomic	5	A/G	Ser191/Phe191
rs183742907	genomic	5	A/C	Ala195/Ser195
rs116376263	cDNA	5	A/T	Asp207/Glu207
rs151245420	cDNA	5	C/T	Ser217/Gly217
rs191733133	genomic	5	C/T	Arg222/His222
rs188639368	genomic	5	A/G	Ile226/Thr226
rs143744963	genomic	5	C/T	Ile226/Val226
rs117446494	cDNA	5	C/T	Asn232/Ser232
rs148749882	cDNA	6	A/G	Ser235/Pro235
rs149300466	cDNA	6	A/G	Pro240/Ser240
rs138921267	cDNA	6	C/T	Val243/Met243
rs146268123	cDNA	6	A/G	Phe250/Leu250
rs148477675	cDNA	7b	A/G	Ala310/Val310

^a Exon numbering as shown in Fig. 1.

Table 2. Single nucleotide polymorphisms of the renalase gene characterized for their association with pathological conditions.

SNP accession no.	Location ^a	Alleles	MAF ^b	Diseases and risk-associated allele	Ref.
rs2576178	5' flanking region	G/A	0.46	Essential hypertension (G); hypertension in end stage renal disease (G); type 2 diabetes (G)	[44,45,67]
rs2296545	Exon 1a	C/G Asp37/Glu37	0.44	Essential hypertension (C); hypertension in type 2 diabetes (C); cardiac hypertrophy, dysfunction and ischemia (C)	[33,44,45]
rs2765446	Intron 3-4	C/T	0.46	n. s. ^c	[44]
rs2437871	Intron 3-4	A/C	0.46	Type 2 diabetes (A)	[68]
rs11202776	Intron 4-5	C/T	0.12	n. s. ^c	[44]
rs1648512	Intron 5-6	A/G	0.32	n. s. ^c	[44]
rs10887800	Intron 5-6	A/G	0.50	Hypertension in end stage renal disease (G); stroke (G)	[45,67]
rs1035796	Intron 7a-7b	C/T	0.47	n. s. ^c	[44]
rs2114406	3' flanking region	A/G	0.22	n. s. ^c	[44]

^a Intron number numbered according to adjacent exons as shown in Fig. 1. ^bMAF, minor allele frequency; ^cn. s., no significant correlation with the considered pathological conditions.

Table 3. Outline of the structural superfamily of *p*-hydroxybenzoate hydroxylase.^a

Superfamily	Family	Representative members	Flavin binding	Catalyzed reaction	Metabolic function	Ref.
PHBH	PHBH-like enzymes	PHBH	Noncovalent	Hydroxylation of <i>p</i> -hydroxybenzoate to yield protocatechuate	Catabolism of aromatic acids in <i>Pseudomonas</i>	[50]
		Monooxygenase PhzS	Noncovalent	Hydroxylation of 5-methyl-phenazine-1-carboxylate to yield pyocyanin	Pyocyanin biosynthesis in <i>Pseudomonas</i>	[73]
		Dihydroxypyridine hydroxylase	Noncovalent	Hydroxylation of 2,6-dihydroxypyridine to yield 2,3,6-trihydroxypyridine	Nicotine degradation in <i>Arthrobacter</i>	[74]
	L-amino acid oxidase-like enzymes	L-amino acid oxidase	Noncovalent	Oxidation of L- α -amino acids to the corresponding α -keto acids	Amino acid catabolism	[75]
		MAO	Covalent bond between FAD 8-methyl and S γ of a Cys residue	Oxidation of monoamines to the corresponding aldehydes	Catabolism of catecholamines and other monoamines	[76]
		N,N-dimethylglycine oxidase	Covalent bond between FAD 8-methyl of and N ϵ of a His residue	Oxidative demethylation of N,N-dimethylglycine to yield sarcosine	Choline and 1-carbon metabolism	[77]
		Polyamine oxidase	Noncovalent	Oxidation of secondary amino groups of polyamines with hydrolysis of resulting imines	Spermidine and spermine catabolism	[78]
		Lysine-specific histone demethylase 1	Noncovalent	Oxidative demethylation of histone mono and di-methylated Lys residues	Regulation of gene expression by nucleosome demethylation	[79]

D-amino acid oxidase-like enzymes	D-amino acid oxidase	Noncovalent	Oxidation of D- α -amino acids to the corresponding α -keto acids	Amino acid catabolism	[80]
	Monomeric sarcosine oxidase	Covalent bond between FAD 8-methyl and S γ of a Cys residue	Oxidative demethylation of sarcosine to yield glycine	Choline and 1-carbon metabolism	[81]
	Glycine oxidase	Noncovalent	Oxidation of D- α -amino acids to the corresponding α -keto acids	Amino acid catabolism	[82]
UDP-galactopyranose mutase-like enzymes	UDP-galactopyranose mutase	Noncovalent	Interconversion between UDP-galactopyranose and UDP-galactofuranose	Biosynthesis of cell wall precursors in Gram-negative bacteria, fungi and protozoa	[83]
	Poly-unsaturated fatty acid isomerase	Noncovalent	Double-bond isomerization of polyunsaturated fatty acid	Biosynthesis of conjugated linoleic acids	[84]

^a This classification of the members of the PHBH superfamily is adapted from that of SCOP, Structural Classification of Proteins [85].

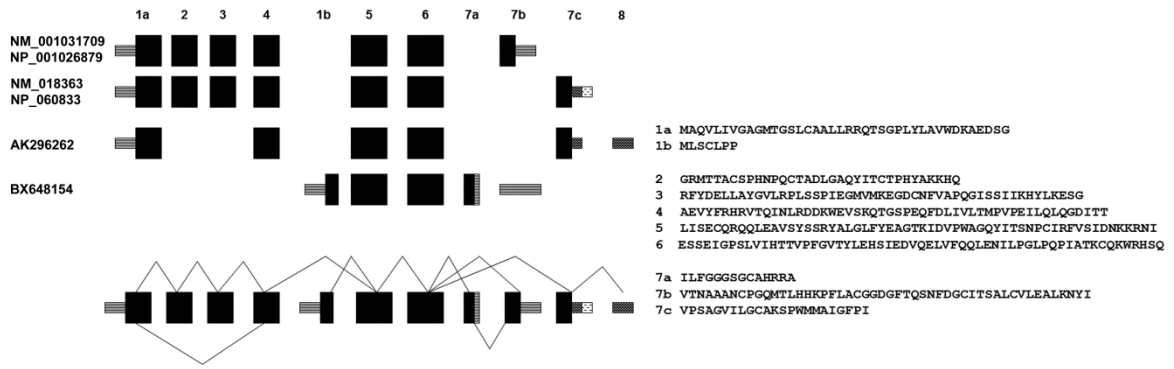


Figure 1

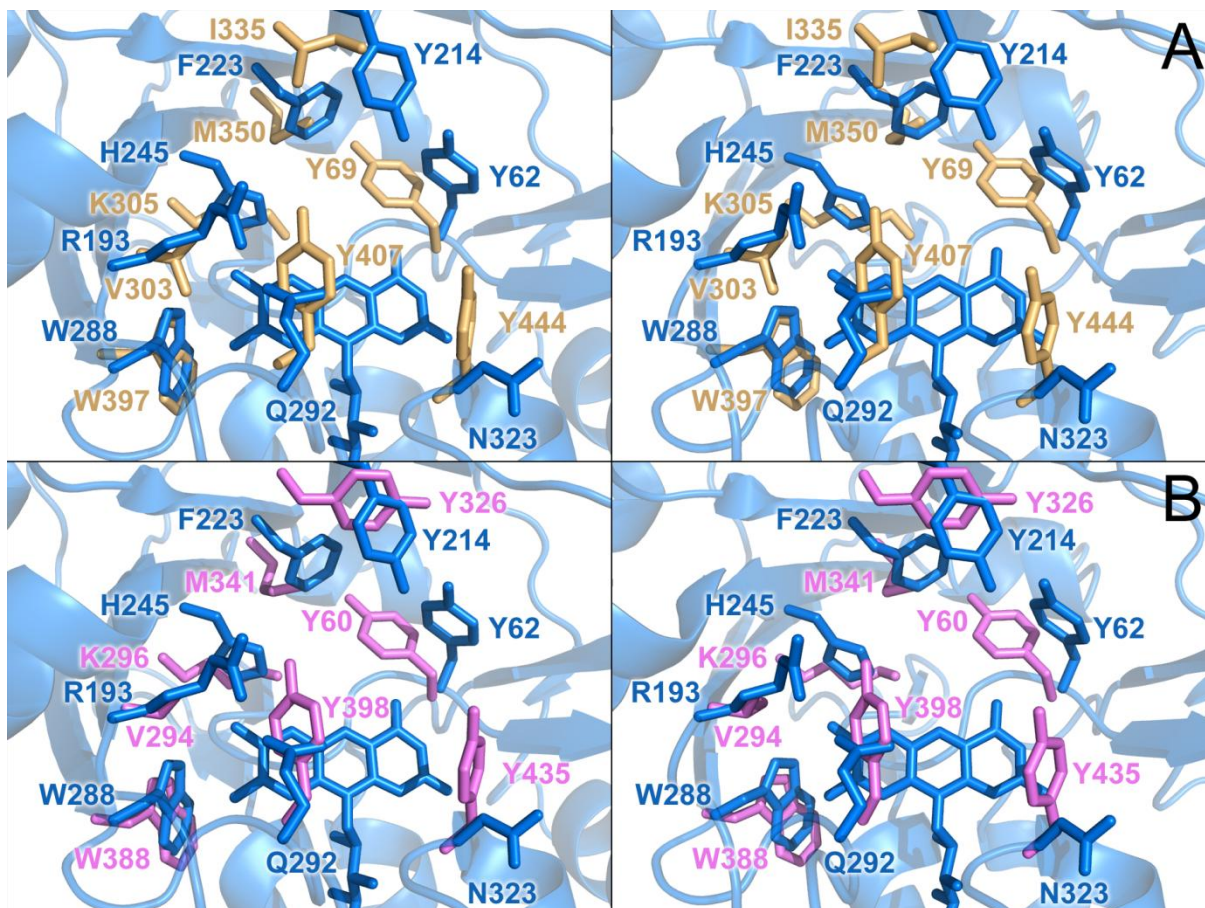


Figure 2

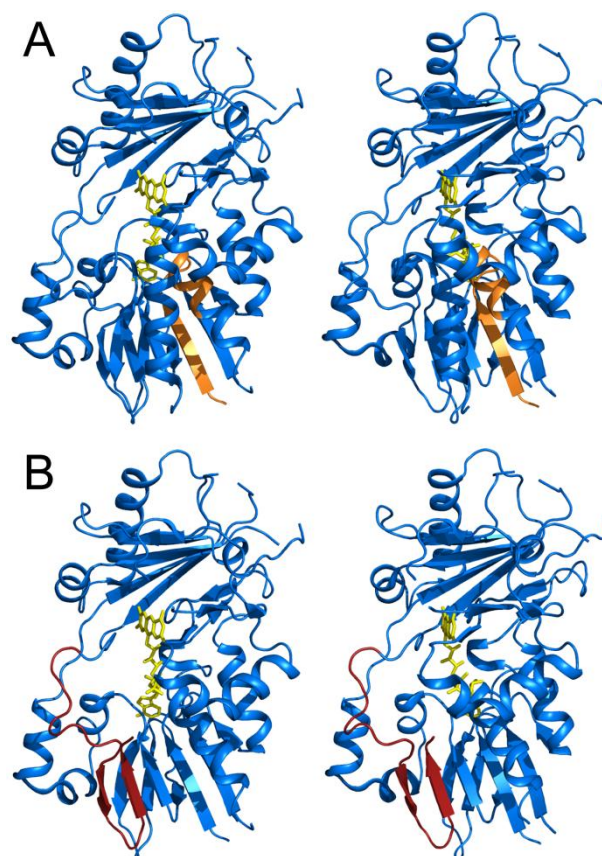


Figure 3