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REVIEW

Renalase, a New Secretory Enzyme Responsible for Selective Degradation of Catecholamines: Achievements and Unsolved Problems

A. E. Medvedev*, A. V. Veselovsky, and V. I. Fedchenko

Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, Pogodinskaya ul. 10, 119121 Moscow, Russia; E-mail: professor57@yandex.ru

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Abstract—Renalase is a recently discovered secretory enzyme responsible for selective degradation of blood catecholamines. The review summarizes literature data on expression of this enzyme and on its structure and functions. Special attention is paid to unsolved and questionable problems including: 1) prediction of the presence of FAD in the protein structure based on amino acid sequence similarity of renalase with known FAD-dependent enzymes; 2) identity of plasma and urinary renalase; 3) mechanism underlying conversion of inactive renalase into the active form.

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In 2005, the *Journal of Clinical Investigation* published the first report of a group of scientists from Yale University about discovery of a new enzyme responsible for degradation of circulating plasma catecholamines [1]. This attracted much interest of the scientific community [2-5]. Some scientists dubbed renalase a catecholamine-metabolizing hormone (!?) [2], whereas others were rather skeptical because the enzyme activity in human blood was too low to make any serious contribution to regulation of the level of circulating catecholamines [3]. Nevertheless, certain research groups started to analyze features of the renalase gene in population studies of essential hypertension [4] and to use this enzyme as a marker responsible for regulation of blood plasma catecholamines [5].

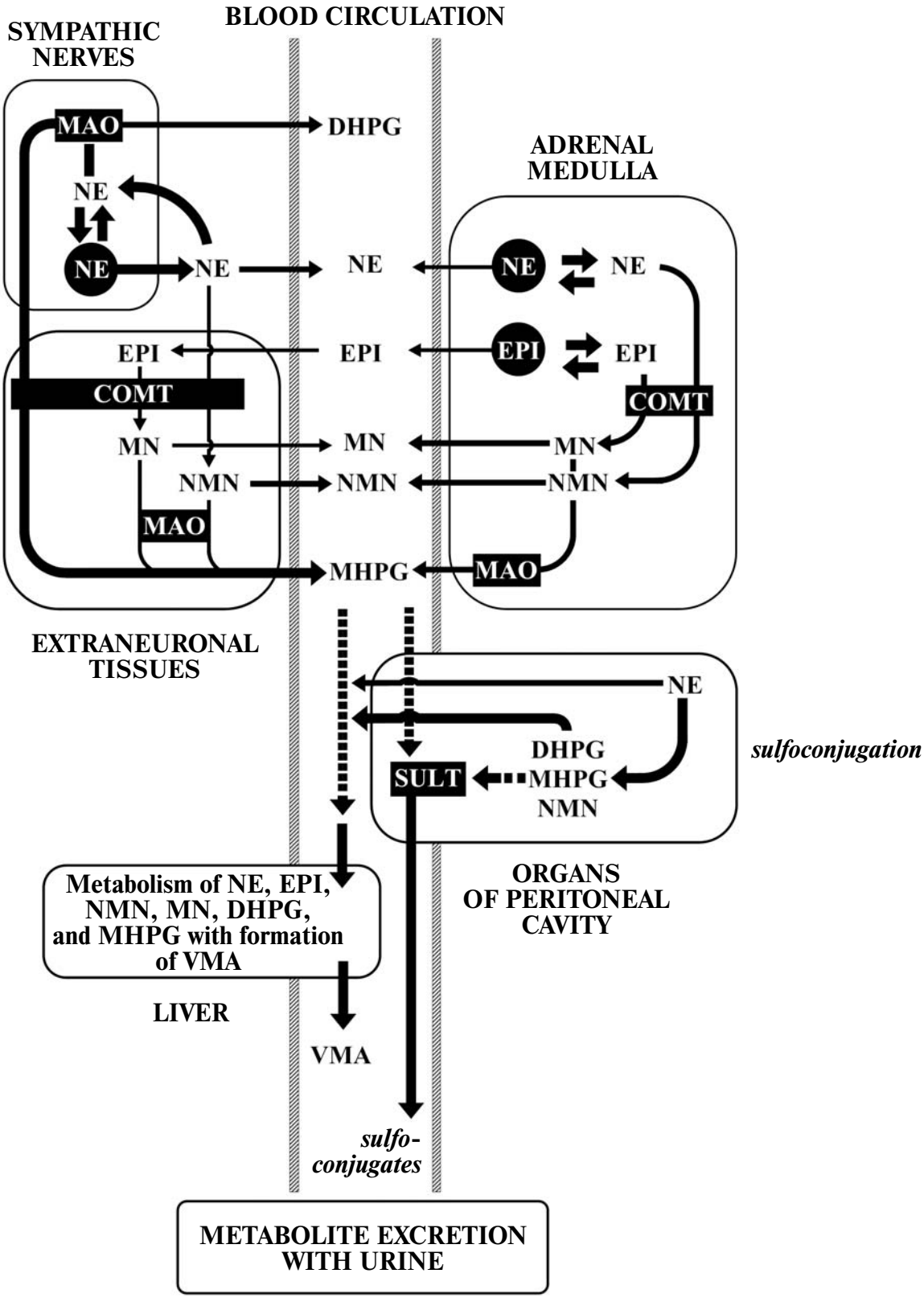
Although at the moment the existence of this new enzyme raises more questions than clear answers, the need for analysis of accumulated information on this problem becomes increasingly evident.

WHY IS THE ENZYME CIRCULATING IN BLOOD IMPORTANT?

Taking a look at the classical scheme of catecholamine metabolism (figure), one can see that all enzymes involved in catecholamine metabolism [6] are localized inside cells. Consequently, all catecholamines reaching the circulation will readily exhibit regulatory (including unwanted) effects on various organs and systems.

It should be noted that blood plasma of many animal species contains semicarbazide-sensitive amine oxidase (SSAO). This copper-containing enzyme (earlier classified in the Enzyme Nomenclature as 1.4.3.6 and now having entry number 1.4.3.21) catalyzes oxidative deamination of primary amines with formation of corresponding aldehydes, hydrogen peroxide, and ammonia. It contains TOPA (trihydroxyphenylalanine) quinone as a cofactor [7, 8]. Methylamine, aminoacetone, and also benzylamine and 2-phenylamine are preferential (and obviously physiological) substrates of SSAO [3, 7-9]. In addition this enzyme catalyzes (although less effectively) oxidation of dopamine and noradrenaline, but not adrenaline [3]. SSAO level in human plasma is very low and *in vitro* incubation with exogenously added catecholamines has minor influence on their levels [3]. In transgenic mice SSAO overexpression did not influence blood pressure after adrenaline administration in comparison with wild type animals [10]. Administration of Geraniin, a hydrolyzable tannin purified from aqueous acetone extracts of *Phyllanthus urinaria* and exhibiting properties of antioxidant and SSAO inhibitor, caused antihypertensive effect [11]. All these data provide

* To whom correspondence should be addressed.



Scheme of catecholamine metabolism (modified from [6]). Abbreviations: MAO, monoamine oxidase; COMT, catechol-O-methyl transferase; DHMA, 3,4-dihydroxymandelic acid; DHPG, 3,4-dihydroxyphenylglycol; MHPG, 3-methoxy-4-hydroxyphenylglycol; VMA, vanillylmandelic acid; HVMA, homovanillinic acid; SULT, sulfotransferase; NE, noradrenaline (norepinephrine); EPI, adrenaline (epinephrine); NMN, normetanephrine, MN, metanephrine

convincing evidence that SSAO is not involved in degradation of circulating catecholamines *in vivo*.

A possible contribution of the copper transport protein ceruloplasmin, which can be involved in catecholamine oxidation *in vitro* [12, 13], is obviously negligible under physiological conditions [12, 14]. It should also be noted that amine oxidase activity of ceruloplasmin does not result in hydrogen peroxide formation [3, 12], which is often used for estimation of amine oxidase activity of blood plasma.

DISCOVERY OF RENALASE

Discovery of renalase represents an impressive illustration of effective use of post-genomic technologies. The authors of [1] started their search for a gene coding a previously unknown secretory protein that would satisfy the following criteria: 1) sequence similarity/identity to known proteins should not exceed 20%; 2) a protein of interest has to contain a signal peptide sequence typical for secretory proteins; and 3) lack of a transmembrane domain (which is detected in proteins fixed in the plasma membrane and exposed into the extracellular space). Analyzing all known clones published by that time within the MGC project (Mammalian Gene Collection Project), the authors selected 114 human candidate genes satisfying the selected criteria. The study of expression pattern for each gene revealed one clone with robust and preferential expression in human kidney. The major band of 1.5 kb (MGC12474; GenBank accession number BC005364) was also detected in heart, skeletal muscle, kidney, and liver. Two additional (and weaker) bands were also detected: one (2.4 kb) was found in skeletal muscle, whereas the other one (1.2 kb) was found in kidney and liver. After sequence analysis of MGC12474 cDNA in the Human Genome Project database, the authors identified the exon–intron structure of the human gene, which they named renalase.

STRUCTURE AND EXPRESSION OF RENALASE

According to information available in GenBank, the renalase gene located on chromosome 10 at q23.33 contains seven exons (NC_000010) and has two transcription variants (1 and 2). The longer variant mRNA (1) encodes a protein of 342 amino acid (aa) residues (MN_001031709.2) with a predicted molecular mass of 37.85 kDa and p*I* of 6.06; the shorter variant mRNA (2) encodes a 315-aa protein (MN_0018363.3) with a predicted molecular mass of 34.95 kDa and p*I* of 6.27. The major differences in the amino acid sequence are determined by exon 7.

Using antirenalase antibodies, a 37.8-kDa protein has been detected in the same rat tissues that contain renalase mRNA [1]. Primary analysis of renalase gene expression in human tissues has shown its preferential expression in renal glomeruli, proximal tubules, and also in cardiomyocytes, liver, and skeletal muscles [1]. Recently, other authors also revealed renalase expression in peripheral nerves, adrenals, and human adipose tissues [15, 16]. They identified several splicing variants of the renalase gene, which can be tissue specific and reflect particular function of renalase in these tissues.

In the deduced (by cDNA) amino acid sequence [1] the *N*-terminal signal peptide, typical for secretory proteins (residues 1-17 according to the signal peptide database, http://www.signalpeptide.de/index.php), so-called "FAD-binding site" (residues 4-35), which partially overlaps with the signal peptide and represents in reality a dinucleotide-binding site (see below), and the amine oxidase domain, including residues 75-339, have been recognized. The amino acid sequence of renalase significantly differs from monoamine oxidases A and B (EC 1.4.3.4), the main FAD-dependent enzymes catalyzing oxidative deamination of catecholamine in the body. For example, renalase shares only 13.2% identity with MAO A [1].

In 2008, Wang et al. [17] identified a human renalase homolog in mouse. They named this enzyme monoamine oxidase C (after MAO A and MAO B), and the gene encoding the enzyme has been included in the NCBI GenBank database as *mMAO-C* (accession number: DQ788834). The renalase gene is located on mouse chromosome 19C1, and its coding region spans seven exons. The coding region includes 1100 bp with an open reading frame of 1026 bp encoding a protein of 342 aa with predicted molecular mass of 37.6 kDa and p*I* of 7.9. Mouse renalase is predominantly expressed in the kidney and testicle and also in liver, heart, and embryo (12.5 days), and it is weakly detectable in brain and skeletal muscle [18]. In the structure of this enzyme also deduced from its nucleotide sequence, the authors also identified a typical signal peptide, an FAD-binding site, and the amine oxidase domain.

CATALYTIC AND REGULATORY PROPERTIES OF RENALASE

The catalytic and regulatory properties of renalase have been elucidated for the human enzyme only. It appears that renalase is an amine oxidase that exhibits its catalytic activity towards the catecholamines dopamine, noradrenaline, and adrenaline [1]. The enzyme is basically inactive using other physiologically active amines as substrates: serotonin, tyramine, benzylamine, methylamine, and spermidine. The mechanism of reaction catalyzed by this enzyme remains unclear; it is only known that incubation of renalase with potential substrates results in formation of hydrogen peroxide, which has been used for evaluation of activity of this enzyme.

Since expression of catalytically active recombinant enzyme in *Escherichia coli* cells has been detected only in

the presence of 0.1 μM FAD, this fact is considered as evidence that renalase is a FAD-dependent enzyme [1]. Pargyline and clorgyline, mechanism-activated MAO inhibitors forming a covalent adduct with the flavin cofactor, did not inhibit renalase activity [1]. Thus, renalase differs from known FAD-dependent amine oxidases by both substrate specificity and sensitivity to the diagnostic inhibitors.

In blood plasma of healthy volunteers renalase activity was almost undetectable. Analyzing the experimental data available in the first publication on renalase [1], Boomsma and Tipton [3] concluded that catecholamine oxidation by renalase *in vitro* is a rather slow process even at non-physiologically high concentration of substrates. In subsequent studies on rats [18], plasma renalase was also basically undetectable. However, after intravenous infusion of catecholamines (dopamine or adrenaline) to anesthetized catheterized rats a several-fold increase in plasma renalase activity was observed after 1 min. Since antirenalase antibodies blocked this effect, they concluded that the increase in amine oxidase activity could be attributed to renalase activation. Repeated administration of adrenaline to animals resulted in the 2.5-3-fold increase in the renalase level observed after 15 min [18].

In experiments with H9C2 cells (a rat heart myocyte cell line), incubation with 20 μM dopamine for 12 h caused a 10-fold increase in renalase mRNA [18]. This suggests that substrate can stimulate expression of the renalase gene.

A catalytically active enzyme was isolated from urine of healthy volunteers by means of ammonium sulfate fractionation followed by subsequent affinity chromatography on an agarose-antirenalase affinity column [1]. The purified renalase preparation contained two proteins, one with the expected size (approximately 35 kDa) and another larger one (67-75 kDa). The authors suggest that the latter protein represents a dimerization (or aggregation) product of the 35-kDa protein. The specific activity of urinary renalase was two orders of magnitude higher than that of plasma renalase. Clear differences in catalytic activity of these enzymes could be attributed to various reasons: 1) origin of urinary renalase is unrelated to plasma renalase; 2) catalytic properties of a dimer differ from those of the monomer; 3) blood plasma contains some inhibitor of renalase activity.

The addition of a plasma aliquot to samples containing urinary renalase caused 80% inhibition of the enzyme activity. In model experiments, albumin alone had no effect on urinary renalase activity. These data indicate that plasma renalase exists in an inactive form called prorenalase [1]. This is an inappropriate term because biochemically the prefix "pro" is applicable to proteins/enzymes to define a higher molecular mass precursor that is converted into a mature protein via limited proteolysis. The data on inhibition of purified enzyme activity by blood plasma can be considered as evidence for existence of some inhibitor that binds to renalase in the absence of a substrate and inactivates the enzyme; a sharp increase in concentrations of circulating catecholamines results in inhibitor displacement and reactivation of the enzyme.

ROLE OF RENALASE

Although there are clear (and quite understandable) gaps in current knowledge on renalase, the above-considered data suggest that renalase is a circulatory enzyme that is responsible for selective degradation of catecholamines circulating in blood plasma and a decrease in blood pressure. Thus, one can expect that decreased formation and secretion of this enzyme would result in increased blood pressure.

Indeed, it has been reported that the development of experimental hypertension in Dahl rats with inherited predisposition to salt-sensitive arterial hypertension was accompanied by renalase deficiency [19]. Subtotal nephrectomy inducing the development of renal insufficiency also decreased the content of circulating renalase [20]. Decreased expression of renalase (both mRNA and protein) was also found in hearts of rats subjected to such procedure during the neonatal period (24-48 h) [5]. These animals also had increased level of plasma noradrenaline [5]. Renalase inhibition by antisense RNA resulted in increased basal blood pressure and hyperergic response to adrenergic stress [19].

Renalase knockout mice were characterized by increased level of arterial pressure and susceptibility to myocardial ischemia [20]. Administration of human recombinant renalase to rats decreased arterial pressure and heart rate [18].

Using three single nucleotide polymorphisms (SNP), association between the renalase gene and the development of essential hypertension was recognized in the northern Han Chinese population [4]. Decrease in renalase secretion by the kidney was found in patients with neurogenic hypertension (i.e. hypertension determined by the CNS rather than by renal pathology). However, this observation was performed in a small group of patients [21].

Although all these data suggest the involvement of renalase in regulation of the cardiovascular system, it should be noted that numerous enzymological aspects of functioning of this enzyme still remain to be clarified. Moreover, interpretation of results in this field does not give deeper understanding of the basic principles of the structure–functional organization of this enzyme.

PROBLEMS

Below we consider the three most important problems that need to be solved first of all.

1. Is renalase a FAD-dependent enzyme? The conclusion that renalase is a FAD-dependent enzyme is based on two indirect facts: the amino acid sequence of renalase contains a region typical for FAD-dependent amine oxidases, MAO A and MAO B [1, 18]; in the absence of exogenous FAD in the cultivation medium cells did not synthesize catalytically active renalase [1].

In fact, the deduced amino acid sequence of renalase contains the so-called Rossmann fold, a dinucleotide-binding region that consists of consecutive β-sheet, α-helix, and another β-sheet (β_1-α-β_2). Its characteristic feature is the presence of a sequence motif Gly-X-Gly-X-X-Gly (where X is any aa) between the first sheet and the helix. This fold basically determines noncovalent interaction between the protein and the ribose-phosphate part of dinucleotide(s) but does not determine the type of dinucleotide. Interestingly, the authors who deduced the amino acid sequence of FAD-dependent MAO A and MAO B, were more cautious in their conclusions [22]. Analyzing the *N*-terminal site of the amino acid sequence of MAO A and MAO B, they indicated the existence of AMP-binding site (residues 15-29 and 6-20 in MAO A and MAO B, respectively), which was characterized by extensive sequence identity in several other flavoproteins including lipoamide dehydrogenase, glutathione reductase, thioredoxin reductase, D-amino acid oxidases, etc. [22].

According to the Structural Classification of Proteins (SCOP) database there are three superfamilies containing such fold, and these can interact with FAD and NAD(P). Search in the PDBeMotif database (http://www.ebi.ac.uk/pdbe-site/pdbemotif/start) using the search query "secondary-structure-pattern: ELHLE; pattern: GXGXXG; ligand: NAD" revealed the presence of 130 structures with the Rossmann folds and NAD in the PDB, whereas the same query but for the presence of FAD yielded 142 structures.

Thus, the presence of the pattern Gly-X-Gly-X-X-Gly in an amino acid sequence cannot be considered as evidence for the presence of FAD in an enzyme. This suggests that the presence of FAD in the renalase structure has to be demonstrated by direct experiments.

However, it should be noted that a carefully performed bioinformatic analysis suggests the possible presence of noncovalently bound flavin in renalase (Veselovsky et al., prepared for publication). Our analysis of the mouse renalase sequence using the SUPERFAMILY hidden Markov model library [23] suggests that renalase can be assigned to the superfamily of proteins containing the FAD/NAD(P)-binding domain, to the family of FAD-dependent reductases containing the domain in the *N*-terminal region (FAD/NAD(P)-binding domain, FAD-linked reductases, *N*-terminal domain). Interestingly, MAO also belongs to this superfamily (and the family). Our search revealed that *Pseudomonas syringae* q888a4 oxidoreductase (PDB code 3kkj), a flavin-dependent amine oxidase with known spatial structure, shares 28% sequence identity with renalase. This is at the lowest identity limit (30% [24]) required for reliable modeling of spatial structure of proteins by homology. In other words, there is a rather high probability that such identity of amino acid sequences implies identity of spatial structures of two compared proteins [24]. Thus, it is reasonable to suggest that the spatial fold of renalase is similar to that of q888a4 oxidoreductase.

Expression of catalytically inactive renalase in cells cultivated in the absence of FAD reported in the first publication on renalase [1] also requires further investigation. It remains unclear whether lack of FAD influences biosynthesis of renalase apoprotein, or the absence of the cofactor prevents formation of active holoenzyme. In this connection, it should be mentioned that ten years ago Nandigama and Edmondson expressed mutant human MAO A (in which Cys406 responsible for covalent FAD binding was replaced by Ala; C406A) in *Saccharomyces cerevisiae* cells sensitive to riboflavin deficiency [25]. In the presence of riboflavin or its analogs, the cells produced catalytically active enzyme. In the absence of riboflavin, the cells synthesized inactive apoprotein, which, however, was correctly inserted into its "working place" in the outer mitochondrial membrane, exhibited catalytic activity, and was sensitive to the specific inhibitor clorgyline after FAD addition [25]. Similar results have been obtained for other flavoproteins containing covalently bound flavin [26-28].

Since whether addition of FAD reactivates renalase expressed in *E. coli* BL21 [1] cultivated in a FAD-free medium has not investigated, the role of FAD in renalase functioning remains to be clarified.

2. Are the urinary and plasma renalases the same enzyme? If we accept the viewpoint and arguments of the authors of [1] that renalase is a FAD-dependent amine oxidase, a reasonable question concerning identity of plasma and urinary renalases arises. A proteomic study of urinary proteins of healthy individuals performed after the first publication on renalase resulted in identification of more than 1500 proteins [29]. However, in the group of proteins involved into amine metabolism, only diamine oxidase was identified as the γ-subunit of the amiloride-sensitive sodium channel exhibiting diamine oxidase activity [30]. Diamine oxidase catalyzes oxidative deamination of the diamines putrescine and cadaverine and also of histamine. Earlier activity of this enzyme, which contains the TOPA-quinone cofactor [7], had already been detected in urine of patients with impaired renal functions [31]. Interestingly, diamine oxidase was not found among urinary proteins with molecular masses corresponding to their intact forms [29]. In other words, diamine oxidase "transfer" from blood to urine was obviously accompanied by changes in its molecular mass. In this connection it is reasonable to note that the molecular mass of homogenous alpha-amylase purified from human

Table 1. Oxidoreductase enzymes identified during human urine proteome analysis [29]

	Name
	Dehydrogenases
1	Alcohol dehydrogenase
2	Lactate dehydrogenase A
3	Lactate dehydrogenase B
4	NAD(P)H dehydrogenase, quinone 2
5	NAD^+-dependent glycerol 3-phosphate dehydrogenase (cytoplasmic)
6	NAD(P)H-dependent leukotriene B4 12-hydroxydehydorgenase
7	Malate dehydrogenase
8	NADP-isocitrate dehydrogenase
9	Quinone oxidoreductase
10	GAPD I
11	Retinal dehydrogenase
12	6-Phosphogluconate dehydrogenase (decarboxylating)
	Reductases
1	Dihydropteridine reductase
2	Glutathione reductase
3	Aldose reductase
4	L-Xylose reductase
5	Glyoxylate reductase/hydroxypyruvate reductase
6	Flavin reductase
7	Carbonyl reductase, NADPH dependent
	Oxidases
1	Diamine oxidase
2	Glutathione peroxidase
3	Pyridoxine 5′-phosphate oxidase
4	Prenylcysteine oxidase

urine (45 kDa) [32] was significantly lower than that of isoenzymes of pancreatic amylase (55.8 kDa) representing the major "source" of urinary "alpha"-amylase [33]. In addition, it should also be noted that proteolysis (or oxidation of SH-groups) can cause spontaneous transformation of NAD-dependent xanthine dehydrogenase (EC 1.17.1.4) into xanthine oxidase (EC 1.17.3.2) [34].

Although renalase has not been included into the enzyme nomenclature, it appears that it might be included in the class of oxidoreductases. In this connection, the above-mentioned proteomic study of human urine identified 23 oxidoreductases (Table 1), and only six of them were FAD-dependent enzymes. In all these enzymes FAD was noncovalently bound to and easily dissociated from the proteins (Table 2).

Thus, it is possible that catalytic activity of urinary renalase does not depend on the presence of FAD. Since the experimentally determined molecular mass of the urinary enzyme (35 kDa) [18] is lower than the calculated molecular mass (37.8 kDa) [1, 17, 18], which includes the signal peptide overlapping with the Rossmann fold, such possibility really exists. In addition, there is the second transcription variant of the renalase gene (see above), which encodes a protein with calculated molecular mass of ~35 kDa.

We suggest that properties of plasma and urinary renalases are not identical, and purification of the urinary enzyme by means of antirenalase antibodies might reflect the presence of the same epitopes. It is also possible that the urinary enzyme represents either a product of intravascular "processing" or the protein product of the other spice variant considered above.

3. How does conversion of inactive renalase into catalytically active enzyme occur? For analysis of possible mechanisms involved in conversion of inactive renalase into the catalytically active enzyme, we should take into consideration data on the increase in plasma renalase activity after catecholamine infusion into rats [18] and higher activity of urinary renalase existing as monomer and (obviously) dimer, which decreased after addition of a plasma aliquot [1, 18]. These at first glance independent data might be explained from a unitary position if we propose the existence of endogenous inhibitor(s) of renalase in plasma. If such inhibitor actually exists, the increase in renalase activity in response to a sharp increase in circulating catecholamines might well be explained by dissociation of a renalase–inhibitor complex followed by possible oligomerization (dimerization) of the enzyme. There are examples illustrating existence of inactive enzyme–inhibitor complexes in plasma, but mainly for various proteases [43, 44].

The other possible explanation of increased renalase activity in plasma *in vivo* can be associated with additional release of renalase molecules from kidneys. Such examples are also known in the literature: for example, treatment of perfused epididymal white adipose tissue

Table 2. Some properties of flavin oxidoreductases identified during human urine proteome analysis [29]

Enzyme	EC	Presence of bound FAD(FMN)	Commentaries
Quinone oxidoreductase	1.6.5.2	+	FAD noncovalently bound in subunit contact region [34]
Retinal dehydrogenase	1.2.1.36	±	FAD addition to purified rat enzyme increased activity only slightly (by 20%) [35]. No data are available for human enzyme (www.brenda-enzymes.org/php/result_flat.php4?ecno=1.2.1.36)
Glutathione reductase	1.8.1.7	+	treatment with ammonium sulfate in acidic medium removed FAD from the enzyme [36, 37]
Flavin reductase	1.5.1.30	+	flavin cofactor was not determined in human enzyme purified from erythrocytes [38]
Pyridoxine 5′-phosphate oxidase	1.4.3.5	(+)	purified enzyme (including human recombinant enzyme) contains tightly bound FMN that dissociated during dialysis against 2 M KBr in phosphate buffer [39, 40]
Prenylcysteine oxidase	1.8.3.5	+	mature enzyme contains noncovalently bound flavin [41]

Note: Symbol "±" indicates lack of such data for the human enzyme.

with nitric oxide donors resulted in release of lipoprotein lipase involved in metabolism of circulating blood lipoproteins [45].

Analysis of publications on renalase demonstrates current achievements in studies of expression of this enzyme. Detection of renalase mRNA suggests a wide distribution of this enzyme in humans (kidney, heart, skeletal muscle, liver, peripheral nerve, adrenal, and adipose tissue) and mouse (kidney, testicle, liver, heart, tissues of 12.5-day-old embryo).

In spite of reports on preparation of antirenalase antibodies, renalase expression in various cell types, and the use of recombinant renalase for decrease of arterial pressure in rats [1, 17], the biochemical properties of this enzyme remain poorly investigated. Structural analysis based on similarity of amino acid sequence of renalase with other proteins does not provide convincing evidence that renalase is an FAD-dependent enzyme.

However, reports on decreased renalase level in patients with end-stage renal disease and the association between the renalase gene and the development of essential hypertension indicate an important but poorly investigate physiological role of this enzyme in the body.

We do believe that the solution of problems considered here will help better understanding of the role of renalase in regulation of catecholamine metabolism, arterial pressure, and possibly other functions.

When this review was already accepted for publication, a paper [46] by Italian authors V. Pandini, F. Ciriello, G. Tedeschi, G. Rossoni, G. Zanetti, and A. Aliverti "Synthesis of human renalase 1 in *Escherichia coli* and its purification as a FAD-containing holoprotein" appeared at the Web-site of the *Journal Protein Expression and Purification* in the section "Papers in press". These authors reported expression of human renalase in *E. coli* cells and its purification to homogeneity. The purified recombinant protein contained noncovalently bound FAD, which easily dissociated in the presence of 0.2% sodium dodecyl sulfate, but did not exhibit monoamine oxidase activity as analyzed by two independent methods. Nevertheless, administration of catalytically inactive renalase to rats decreased arterial pressure. Thus, on one hand, results of the latest study give the direct (and positive) answer to the question whether renalase contains FAD. On the other hand, these results raise another question, whether renalase is a monoamine oxidase? This means that additional studies of this intriguing protein are definitely needed.

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