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Renalase does not catalyze the oxidation of catecholamines

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ABSTRACT

It is widely accepted that the function of human renalase is to oxidize catecholamines in blood. However, this belief is based on experiments that did not account for slow, facile catecholamine autoxidation reactions. Recent evidence has shown that renalase has substrates with which it reacts rapidly. The reaction catalyzed defines renalase as an oxidase, one that harvests two electrons from either 2-dihydroNAD(P) or 6-dihydroNAD(P) to form β-NAD(P)$^+$ and hydrogen peroxide. The apparent metabolic purpose of such a reaction is to avoid inhibition of primary dehydrogenase enzymes by these β-NAD(P)H isomers. This article demonstrates that renalase does not catalyze the oxidation of neurotransmitter catecholamines. Using high-performance liquid chromatography we show that there is no evidence of consumption of epinephrine by renalase. Using time-dependent spectrophotometry we show that the renalase FAD cofactor spectrum is unresponsive to added catecholamines, that adrenochromes are not observed to accumulate in the presence of renalase and that the kinetics of single turnover reactions with 6-dihydroNAD are unaltered by the addition of catecholamines. Lastly we show using an oxygen electrode assay that plasma renalase activity is below the level of detection and only when exogenous renalase and 6-dihydroNAD are added can dioxygen be observed to be consumed.

Introduction

Renalase is widely defined to be a kidney-derived flavoprotein enzyme/hormone whose function is to oxidize circulating catecholamines in order to lower blood pressure and slow the rate of contraction of the heart [1–7]. The initial basis of this claim for renalase activity was an exceedingly small accumulation of hydrogen peroxide in the presence of common neurotransmitter catecholamines when assayed using a generic Amplex Red-based oxidase assay. However, in these initial experiments no account, in the form of a control reaction, was made for background autoxidation [1,5,7–9]. Catecholamines are prone to oxidation in the presence of dioxygen due to their capacity to form semiquinones [10]. The superoxide ion that results rapidly disproportionates to dioxygen and hydrogen peroxide providing a steady non-enzymatic background signal for an Amplex Red assay (Scheme 1). The very low level of H_2O_2 production was subsequently rationalized by proposing that renalase is isolated in a quiescent state that can only be activated in blood in the presence of specific catecholamine neurotransmitters [5,8]. The chemical transformation ultimately claimed for renalase was that oxidized and cyclized aminochrome molecules were the native products and that the reaction was somewhat faster in the presence of NAD(P)H [8]. However, even in these latter studies, no control was made for the addition of the catecholamine or the reductant, despite that NAD(P)H would greatly increase the complexity of the assay by increasing the number of non-enzymatic redox reactions that yield H_2O_2 (Scheme 1). That no control reactions were used to support any of the early catalytic claims emphasizes the overall tenuous foundation on which renalase catecholamine oxidase activity is based.

Boomsma and Tipton were first to register well-reasoned skepticism concerning a catecholamine oxidase role for renalase [11] and were joined by others expressing similar dissent and eventually direct experimental refutation was presented by Pandini et al. [12–14]. However, the flawed initial *in vitro* evidence and counter evidence have been insufficient to quell the passive consensus that the function of renalase is to oxidatively consume catecholamine neurotransmitters from blood. Consequently, the early claims for catecholamine oxidase activity continue to be adduced and correlated with a wide variety of physiological measurements [2–7,9,15–40].

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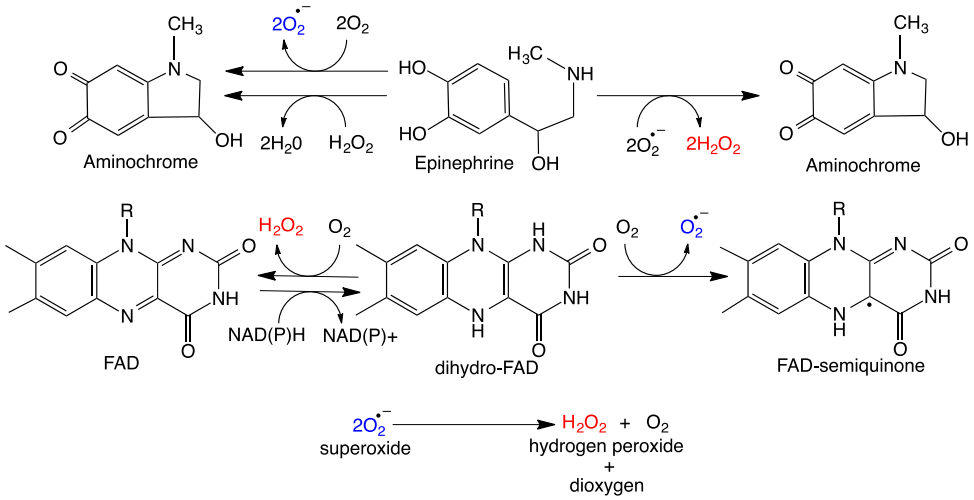


Scheme 1. Non-enzymatic redox reactions in renalase catecholamine oxidase assays.

We recently demonstrated that 2-dihydroNAD (2DHNAD(P)) and 6-dihydroNAD(P) (6DHNAD(P)), both isomers of β-NAD(P)H (4DHNAD(P)), rapidly reduce the FAD[2] coenzyme of renalase (230 s^{-1} for 6DHNAD, 850 s^{-1} for 2DHNAD) and in doing so become oxidized to β-NAD(P)$^+$. The reduced enzyme then reoxidizes by reducing molecular oxygen to form H_2O_2 (2.9×10^3 M^{-1} s^{-1}) (Scheme 2). Moreover, this genuinely catalytic reaction has an apparent metabolic purpose, as both 2DHNAD and 6DHNAD are inhibitory to primary metabolism dehydrogenases [41,42]. The fundamental purpose of this study is corrective; using a verifiably active form of renalase, we demonstrate that no catalytic link exits between renalase and catecholamine oxidation. We show also that renalase is not activated by blood plasma nor is, what we surmise to be, the native activity influenced by the presence catecholamines.

Materials and methods

Materials

Potassium phosphate, L-DOPA, epinephrine hydrochloride, and sodium chloride were obtained from ACROS. Dopamine hydrochloride was purchased from Alfa Aesar. Renalase was expressed and purified according to our previously published methods [43]. β NADH (disodium salt, trihydrate) was obtained from Amresco; β-NAD$^+$ was purchased from Sigma. 6DHNAD was prepared by reduction of β-NAD$^+$ as previously described [41]. Human blood plasma was a gift from Dr. Julie A. Oliver (Biological Sciences Department-University of Wisconsin-Milwaukee). Freshly drawn blood was treated with 10 U/mL heparin (Sagent Pharmaceuticals) to inhibit clotting. The sample was centrifuged at 2200 rpm until blood cell pellet was formed (~15 min). The plasma was separated from the blood cell pellet and hematocrit and stored on ice until needed. Plasma samples were collected, purified, diluted (50% PBS), and used in experiments within 3 h.

HPLC product analysis

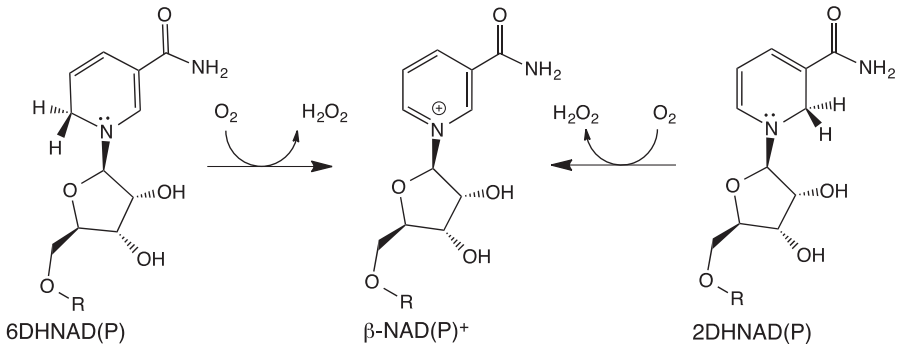
In order to demonstrate the extent to which epinephrine, the catecholamine most often claimed or accepted as a substrate for renalase [1,2,4,5,23], is consumed by renalase, a mixture of β-NADH (50 μM) and epinephrine (150 μM) was prepared in PBS buffer at 25 °C. The sample was then divided into two and renalase (9 μM final) was added to one while the other served the role of a sample age control. A second set of mixtures were prepared in an identical manner, but FAD (9 μM final) was added in place of renalase. All samples were incubated for 10 min prior to ultrafiltration using a 0.5 mL Amicon 10 kDa centrifugal filter (removing renalase from the mixture). 50 μL of each filtrate was then chromatographed by HPLC using a Xterra C18 reversed phase column (4.6 × 150 mm, 3.5 μM particle size) run isocratically at 0.4 ml/min in 10 mM potassium phosphate pH 7.5 coupled to a 600 E HPLC pump and Waters 2487 dual wavelength detector (260 nm and 340 nm). Components eluting from the HPLC column were identified by their characteristic retention time compared to authentic compounds. The chromatograms derived from the renalase and FAD samples were corrected for the minor dilution that occurred with the addition of these components.

Spectrophotometric evidence of neurotransmitter oxidation

Reactions were performed to assess the rates of autoxidation of catecholamines in the presence of renalase (or FAD control) when incubated with a reductant (β-NADH or 6DHNAD). Renalase was prepared by dilution to 40 μM in 2× PBS buffer and mounted onto a onto a Hitech-DX2 (now TgK) stopped-flow spectrophotometer instrument. Reaction mixtures in which renalase (or FAD control) was combined with 100 μM catecholamine (epinephrine, dopamine or L-DOPA) and 15 μM β-NADH (or 6DHNAD control) in water were observed at 458 nm which allows observation of both the FAD redox state and the formation of aminochrome species (λ_{max} ~ 480 nm). All solutions were equilibrated with atmosphere at 25 °C and contained ~250 μM dioxygen.

To assess if catecholamine neurotransmitters (epinephrine, dopamine and L-DOPA) modulate the catalytic behavior of renalase, single turnover reactions with 6DHNAD were performed in the presence and absence of catecholamines with atmospheric dioxygen (~250 μM). Under these conditions the reduction and subsequent reoxidation of the renalase flavin coenzyme can be clearly observed. A native substrate solution, 6DHNAD (30 μM), was prepared in H_2O. This solution was mounted onto the stopped-flow spectrophotometer and mixed with the renalase (40 μM) sample described above and the turnover was observed at 458 nm, the visible absorption maximum for the FAD coenzyme bound to the enzyme. To determine if catecholamine

[2] *Abbreviations used:* HPLC, high performance liquid chromatography; L-DOPA, L-3,4-dihydroxyphenylalanine; Dopamine, 4-(2-aminoethyl)benzene-1,2-diol; Epinephrine, (*R*)-4-(1-hydroxy-2-(methylamino)ethyl)benzene-1,2-diol; FAD, flavin adenine dinucleotide; β-NAD$^+$, oxidized nicotinamide adenine dinucleotide; β-NADH (4DHNAD), reduced nicotinamide adenine dinucleotide; 6DHNAD, 6-dihydronicotinamide adenine dinucleotide; 2DHNAD, 2-dihydronicotinamide adenine dinucleotide ; renalase, human renalase variant 1.



Scheme 2. Proposed native catalytic chemistry of renalase.

neurotransmitters modulate the turnover of renalase, 200 μM of epinephrine, dopamine or L-DOPA was added to the 6DHNAD solution before mixing.

Dioxygen consumption to assess renalase activity in blood and the effect of preincubation

In order to establish the constitutive level of renalase activity in human blood and the effect of preincubation with plasma and/or epinephrine as compared to PBS buffer control, a variety of assays were monitored by dioxygen consumption. Reactions were observed using a Hansatech Oxygraph oxygen electrode and all reaction components were pre-equilibrated with atmospheric dioxygen (~250 μM) at 25 °C. Reactants were added to the following final concentrations: 50% human blood plasma (diluted in PBS buffer), 10 μM epinephrine, 500 nM renalase and 30 μM 6DHNAD. Each assay was initiated by the addition of 6DHNAD, following either a 5 min or 20 min preincubation and then observed for an additional 700 s.

Results

HPLC product analysis

Renalase is claimed to consume neuroactive catecholamines, chiefly epinephrine, in order to lower blood pressure [8]. To demonstrate the extent to which verified active renalase can consume epinephrine in the presence of β-NADH, standardized HPLC chromatograms were collected and compared to controls that contained FAD in place of renalase (Fig. 1). 150 μM Epinephrine was combined with 50 μM β-NADH (that has a β-NAD^+ impurity). The sample was divided into two and renalase added to one. Both samples were then incubated for 10 min (Fig. 1A). Two similar mixtures were prepared as controls but with FAD substituted for renalase (Fig. 1B). Analysis of the chromatograms showed that no significant consumption of epinephrine could be observed in the 10-min window of the incubation. The renalase sample did show significant loss of β-NADH (11 μM) that was not entirely accounted for by a gain in the β-NAD^+ peak area (2 μM). This fraction of the β-NADH was accounted for by the modest dissociation constant for Ren_{ox} β-NADH complex ($K_d \sim 600$ μM) and the ~20-fold increase in the renalase concentration (180 μM) during filtration that together predict the 11 μM retention [41]. The FAD control chromatograms indicate no non-enzymatic chemistry has occurred within the 10 min incubation window. Together these data definitively show that the consumption of epinephrine cannot be detected in the presence of high concentrations (9 μM) of fully active renalase.

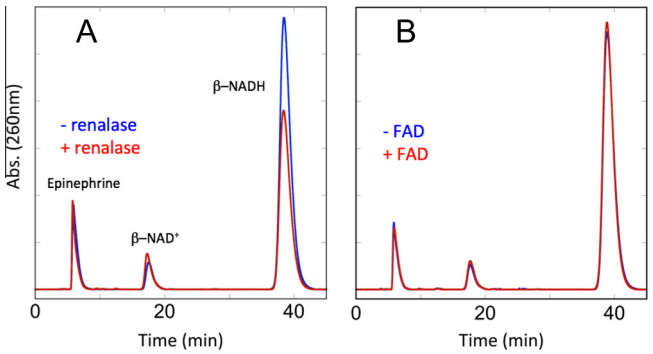


Fig. 1. HPLC analysis of oxidation of epinephrine to adrenochrome by renalase. (A) and (B) were separated using a Xterra reverse phased C18 run isocratically in 10 mM Kpi, pH 7.5 and monitored at 260 nm. (A) The blue line represents the mixture of 150 μM epinephrine and 50 μM β-NADH (4DHNAD) in PBS buffer at 25 °C before the addition of 9 μM renalase and the red trace was taken after the addition of 9 μM renalase and incubation for 10 min at 25 °C; renalase was removed from the sample by centrifugal (Amicon) ultra-filtration prior to injection. (B) The reaction described in A was repeated except FAD was substituted for renalase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Spectrophotometric evidence of neurotransmitter oxidation

Renalase has been reported to oxidize catecholamines to aminochromes (Scheme 1) [9]. It has also been proposed that catecholamines modulate the redox activity of renalase to the extent that the enzyme is largely inactive in the absence of epinephrine [8]. In order to show the capacity of renalase to oxidize catecholamines to form aminochromes, two sets of reactions were performed one in the presence and one in the absence of renalase. Aminochromes are the oxidized and cyclized forms of catecholamines that have a puce color as a result of a broad absorption centered around 480 nm ($\varepsilon_{480} \sim 4020\ M^{-1}\ cm^{-1}$) which provides a simple physical signal to observe their accumulation in solution. The absorption peak of aminochromes is sufficiently broad that at 458 nm, the wavelength of observation used, the extinction coefficient is only 2% lower, permitting simultaneously assessment of the FAD coenzyme and the catecholamine oxidation/cyclization. Three common neuroactive catecholamines were mixed with reductant (β-NADH or 6DHNAD) and renalase (20 μM final) or FAD (20 μM final). In the first control β-NADH was combined with epinephrine, L-DOPA, or dopamine and subsequently mixed with renalase to determine if renalase could enhance the rate of oxidation of the catechols to their associated aminochromes in the presence of NADH. No change in absorbance was observed indicating that renalase does not promote the oxidation and cyclization catecholamines. As a control, FAD was substituted for renalase and the above experiment repeated. This data showed small accumulations

of absorption (epinephrine, 0.0035; DOPA, 0.015; L-DOPA, 0.0025) equating to ~1–3 μM aminochrome after 300 s, indicating that a mixture of β-NADH and free FAD can enhance the rate of catecholamine autoxidation and that renalase actually provides some degree of protection from this chemistry presumably as a result of the active site flavin being inherently unreactive with β-NAD(P)H molecules in order to avoid wasteful diaphorase activity.

In our previous work we have demonstrated that renalase catalyzes rapid oxidation of 6DHNAD(P) and 2DHNAD(P) to β NAD(P)$^+$ where a hydride equivalent is transferred from the nicotinamide base to the renalase flavin coenzyme that then reoxidizes by reducing dioxygen to form hydrogen peroxide (Scheme 2) [41,43,44]. In order to show the extent of the influence of catecholamines on the kinetics of this chemistry, reduction and reoxidaton of the renalase flavin coenzyme that occurs in single turnover with 6DHNAD was observed at 458 nm (Fig. 2B). The data obtained indicate that neither epinephrine, L-DOPA, or dopamine have influence on the catalytic behavior of renalase. The FAD, 6DHNAD (±catecholamines) control for the single turnover reactions where ostensibly the same as those for FAD, β-NADH and catecholamine in Fig. 1A (data not shown).

Dioxygen consumption to assess renalase activity in blood and the effect of preincubation

There are three near constant elements in all scientific articles that pertain to renalase; all claims for activity involve the reduction of dissolved dioxygen, most report or cite that catecholamines are substrates and the majority pivot their investigations on the role of renalase in blood [14,45]. Moreover, the low *in vitro* catecholamine oxidase activity has been claimed to be a consequence of a "prorenalase" form that is largely inactive when separated from some unidentified activator [5,9]. Given that renalase oxidizes 2- and 6DHNAD(P) [41], does renalase exhibit modified catalytic behavior in blood? In order to demonstrate the effects of preincubation with catecholamine and/or blood plasma we conducted a series of assays using an oxygen electrode (Fig. 3). All assays had 6DHNAD added either after a short (5 min) or long (20 min) preincubation.

In Fig. 3, assays 1 & 6, we see that epinephrine is slow to oxidize and does not promote significant consumption of dioxygen prior to or after the addition of 6DHNAD. In addition, assays 1 & 5 indicate

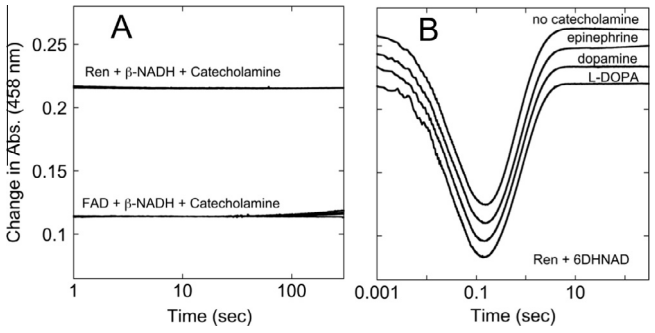


Fig. 2. Catalytic turnover of renalase in the presence of neurotransmitters. (A and B) Renalase single turnover (B) and control(A) stopped-flow spectrophotometer traces were observed at 458 nm for 200 sec. (A) The extent of auto-oxidation of epinephrine was monitored; *top*, stopped-flow spectrophotometer trace of 20 μM renalase with 15 μM β-NADH (4DHNAD) in the absence and presence of 100 μM catecholamine of interest (epinephrine, DOPA, L-DOPA); *bottom*; stopped-flow spectrophotometer trace of 20 μM FAD with 15 μM β-NADH(4DHNAD) and 100 μM of catecholamine of interest (epinephrine, DOPA, L-DOPA). (B) Single turnover of 20 μM renalase with 15 μM 6DHNAD in the absence and presence of 100 μM catecholamine of interest (epinephrine, DOPA, L-DOPA).

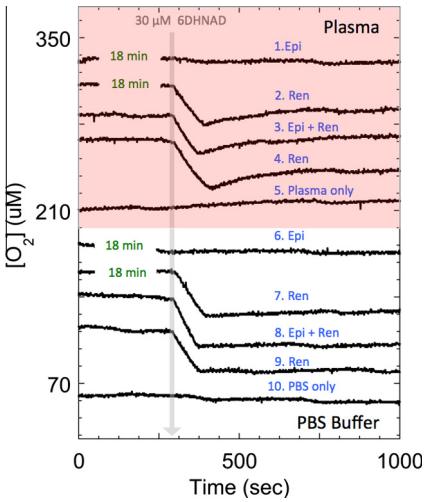


Fig. 3. The effect of catecholamine and plasma preincubation. Renalase activity in 50% blood plasma and PBS buffer was determined by monitoring dioxygen consumption. Reactions were performed by reacting 30 μM 6DHNAD in the absence or presence of 500 nM renalase and 10 μM epinephrine in either PBS buffer or 50% blood plasma. Traces 1, 2, 6, and 7 (from top) were incubated at 25 °C for 20 min prior to the addition of 6DHNAD all other traces were incubated at 25 °C for 5 min before 6DHNAD addition. Traces were aligned so the addition of 6DHNAD appears in the same time position in all reactions. Assays were separated by adding or substracting values for clarity. Only Assay 5 is not plotted on an actual molecular oxygen scale.

that the basal level of renalase activity in blood is sufficiently low to be below the sensitivity of these methods. Using antibody detection, Zbroch et al. determined that the concentration of renalase in plasma was 4 μg/mL (100 nM) [31] approximately one fifth of the exogenous concentration added in assays 2, 3, 4, 7, 8 & 9. That each of these traces show marked dioxygen consumption with added renalase and those without show no dioxygen consumption (assays 1 & 5) suggests that there is very little renalase in blood or that the majority of it is inactive. Comparison of assays 2 & 4 to assays 7 & 9 indicates that preincubation of renalase in plasma does not alter its behavior. Both sets of assays indicate linear consumption of dioxygen that is equimolar to the amount of 6DHNAD added and then cessation of activity. Assays 2, 3 & 4 show recovery of approximately half the dioxygen consumed in the renalase catalytic phase presumably as a consequence of catalase activity in the plasma. Contrary to claims that epinephrine activates renalase [8], neither assay 1 or 6, those preincubated with epinephrine in plasma and PBS respectively, show any evidence of activity, strongly suggesting that the circulating catecholamine does not activate a quiescent form of renalase.

Discussion

The chronology of reported activities for renalase elaborate the erroneous initial claim. In the progenitor article it was proposed that renalase is secreted by the kidney to oxidize circulating catecholamines and that the electrons mobilized are delivered to dioxygen [1]. However, this was surmised only from the data obtained from a generic oxidase assay method and without the use of appropriate controls. Nonetheless, the association of renalase with catecholamines continues to become ever more conflated. Catecholamines are said to regulate the activity, secretion and synthesis of renalase [5]. A variety of complex regulatory feedback pathways have been proposed [5–7,17,23]. Aminochromes are claimed as the native products [9] and the active oxidizing agent is said to be superoxide [8]. However, the extremely low levels of claimed *in vitro* catecholamine oxidase activity has been

cited as a deficit by a number of researchers [11,14,46] and this was then rationalized by invoking "prorenalase" a claimed quiescent form of the enzyme that requires activation [5,6] that occurs only in blood in the presence of a catecholamine [5,8]. It can now be stated with some certainty that this successive affirmative set of claims describes a compounding of an initial scientific deficit; the failure to employ appropriate control reactions to assess catalysis.

The purpose of this study is to clearly demonstrate that renalase is not the third monoamine oxidase. The apparently indelible mark of the early and recent activity claims for renalase must be dispelled if the field is to advance in a purposeful manner. We recently reported two isomeric forms of β-NAD(P)H as substrates for renalase that are clearly catalytically consumed (Fig. 2B) and have redefined renalase as having a house-keeping activity that is unrelated to blood pressure regulation. Renalase functions instead to oxidize reduced forms of nicotinamide adenine dinucleotides that harbor the hydride in non-metabolically accessible positions of the nicotinamide base (positions 2 & 6). We have proposed that this activity exists to relieve inhibition of primary metabolism by these molecules. A principal benefit of having identified native substrates for renalase is that we can now test the validity of previously proposed activities using a form of the enzyme that we have verified is fully active (Fig. 2B) [41]. In sum our data show that renalase does not consume catecholamines (Figs. 1 and 2A), is not kinetically regulated by catecholamines (Fig. 2B), is not isolated in an inhibited form and as such cannot be activated by blood plasma or catecholamines (Fig. 3). In addition, our data suggests that blood has very little if any active renalase (Fig. 3) an observation that is consistent with its newly identified activity that we would suggest has an exclusively intracellular/metabolic role.

Catecholamines form hydrogen peroxide as they oxidize in oxygenated media. That the initial accounts of renalase activity did not employ appropriate control reactions and reported vanishingly low activity undermines the subsequent affirmative claims. The case has also been made that in the absence of genuine substrates, no sample integrity measures have been established and as such there has been no means to discriminate natively folded and active renalase from misfolded inactive but soluble renalase [14]. The vast majority of reports that describe direct use of the enzyme do not indicate the color of the protein (that is conspicuously yellow) or the unique absorption maxima of the flavin in the natively folded enzyme [47]. Nonetheless, in the ten years since its initial discovery, the terms renalase and monoamine oxidase C have become somewhat synonymous [17,48,49] and this has occurred despite sound argument and evidence to the contrary [11,12,14]. In this article we have presented evidence that verifiably active renalase does not catalyze the oxidization of catecholamines.

Conflict of interest

There is no conflict of interest.

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