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Kinetics and Equilibria of the Reductive and Oxidative Half-Reactions of Human Renalase with α-NADPH

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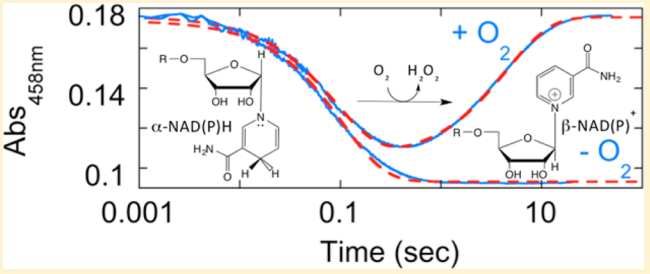
ABSTRACT: Renalase is a recently discovered flavoprotein that has been reported to be a hormone produced by the kidney to down-modulate blood pressure and heart rate. The consensus belief has been that renalase oxidizes circulating catecholamine neurotransmitters thereby attenuating vascular tone. However, a convincing *in vitro* demonstration of this activity has not been made. We have recently discovered that renalase has α-NAD(P)H oxidase/anomerase activity. Unlike most naturally occurring nucleotides, NAD(P)H can accumulate small amounts of the α-anomers that once oxidized are configurationally stable and unable to participate in cellular activity. Thus, anomerization of NAD(P)H would result in a continual loss of cellular redox currency. As such, it appears that the root purpose of renalase is to return α-anomers of nicotinamide dinucleotides to the β-anomer pool. In this article, we measure the kinetics and equilibria of renalase in turnover with α-NADPH. Renalase is selective for the α-anomer, which binds with a dissociation constant of ∼20 ± 3 μM. This association precedes monophasic two-electron reduction of the FAD cofactor with a rate constant of 40.2 ± 1.3 s^{-1}. The reduced enzyme then delivers both electrons to dioxygen in a second-order reaction with a rate constant of ∼2900 M^{-1} s^{-1}. Renalase has modest affinity for its β-$NADP^+$ product (K_d = 2.2 mM), and the FAD cofactor has a reduction potential of −155 mV that is unaltered by saturating β-$NADP^+$. Together these data suggest that the products are formed and released in a kinetically ordered sequence (β-$NADP^+$ then H_2O_2), however, the reoxidation of renalase is not contingent on the dissociation of β-$NADP^+$. Neither the oxidized nor the reduced form of renalase is able to catalyze anomerization, implying that the redox and anomerization chemistries are inextricably linked through a common intermediate.



Reported initially in 2005, renalase was said to be a new renal hormone that is secreted into the blood by the kidney to induce lower blood pressures and slowed heart rate.[1,2] In subsequent studies, it has been reported that mice deficient in renalase have elevated blood pressures and that renalase polymorphisms result in a higher incidence of stroke and diabetes.[3−5] The consensus belief has been that renalase imparts its physiological response by catabolizing circulating catecholamine neurotransmitters (chiefly adrenaline) thereby attenuating vascular tone.[2,6,7] The expression of renalase has been reported in additional tissues[2,8,9] with at least four variants arising from altered splicing of seven exons.[8,9] The X-ray structure of the longest variant, isoform 1 (342 amino acids), was published in 2011, revealing a protein whose structural topology was similar to known flavoprotein oxidases, monooxygenases, and demethylases but whose activity could not be readily discerned from conserved active site residues.[10−15] Given the similarity of the structural fold of renalase to monoamine oxidases A and B, catabolism of catecholamines would appear to be a reasonable, albeit contextual, function for renalase. However, there has been considerable difficulty establishing this activity *in vitro*, leading to the assertion that renalase as isolated is "prorenalase", a form that must be activated in blood by catecholamines or other modifiers.[16] Slow accumulations of adrenochrome in the presence of renalase, epinephrine, and NADPH have been offered as evidence that renalase catalyzes the oxidation of the catecholamines and promotes nucleophilic attack by the side-chain distal amine.[17] The difficulty is that this is a facile, well-established degradative reaction for catecholamines that exhibits measurable rates under mildly oxidizing conditions in the absence of an enzyme catalyst.[18−20] Moreover, the stoichiometry of such a reaction in regard to the fate of the mobilized electrons has not been established.[17] As such, the claim that renalase catabolizes circulating catecholamines has been questioned by a number of researchers.[11,21,22]

We have recently discovered an activity for renalase as a bifunctional α-NAD(P)H oxidase/anomerase.[23] Renalase oxidizes the dihydropyridyl ring of α-pyridine nucleotides, which arise naturally as a small equilibrium component of NAD(P)H solutions, and manages to also epimerize the nicotinamide ribose to form β-$NAD(P)^+$ products (Scheme 1). Importantly, renalase does this chemistry at least 2 orders of magnitude more rapidly than any reported turnover rate for catecholamine oxidation. The electrons acquired from the α-NAD(P)H substrate are initially transferred to the enzyme's FAD cofactor

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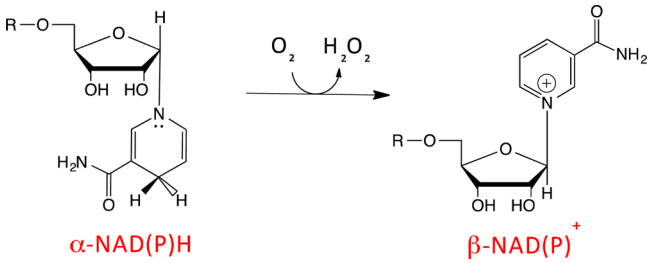
Scheme 1



and then to dioxygen to yield hydrogen peroxide. How and whether this activity is linked to the reported physiological response to renalase has not been addressed, but the only known vasoactive molecule to be consumed or arise from this activity is H_2O_2.[24,25] Coupling NAD(P)H oxidation to α–β anomerization and dioxygen reduction provides a large driving force for this chemistry and suggests that the more pervasive physiological function for renalase is to drive the α/β NAD(P) ratio to zero. In this article, we offer evidence for the kinetics and equilibria of the reductive and oxidative half-reactions of human renalase reacting with α-NADPH.

MATERIALS AND METHODS

Materials. Potassium phosphate, isopropyl-β-thiogalactopyranoside (IPTG), and sodium chloride were obtained from ACROS. Luria–Bertani broth (Lennox) powder was from Fisher Scientific. Kanamycin and β-NADPH were purchased from Alexis. β-NADP$^+$ was purchased from Calbiochem and Axxora. β-NADH, β-NAD$^+$, xanthine, methyl viologen, and epinephrine were from Sigma-Aldrich. Indigo carmine was from ICN. Xanthine oxidase was acquired from Calzyme. Renalase was expressed and purified according to our previously published methods.[23] α-NADPH was from the α/β-NAD(P)H equilibrium mixture (reliably 1.5% (NADPH) and 4% (NADH) α anomers at equilibrium). Anomer equilibrium was achieved by dissolving β-NADPH in PBS or HEPES, pH 7.5, and placing this solution in a sealed vessel at 4 °C for ∼72 h.[26] Prior to using stock solutions, the α-NAD(P)H concentration was determined by dioxygen electrode. Typically 1–2 μM renalase was added to 1–3 mM of the NAD(P)H solution in PBS buffer at 25 °C. The observed amplitude of dioxygen consumption was used to define the fraction of the α-NAD(P)H component.

Quantitation. The concentration of NADPH and NADH solutions were determined using the published 340 nm extinction coefficient of 6200 M^{-1} cm^{-1}.[26] An extinction coefficient for renalase was determined in prior work (ε_{458nm} = 11 330 M^{-1} cm^{-1}).[23] NADP$^+$ and NAD$^+$ solutions were quantified using published 260 nm extinction coefficients.[27]

Reduction Potentials Measurements and β-NADP$^+$ Binding Affinity. Reduction potentials were measured for unliganded oxidized renalase and the Ren$_{ox}$·β-NADP$^+$ complex using the xanthine/xanthine oxidase reduction system of Massey.[28] A Hewlett-Packard 8453 diode-array spectrophotometer was used to monitor the reduction of the enzyme in the presence of indigo carmine ($E°$ = −130 mV). The reaction mixture was placed in an anaerobic cuvette and consisted of 300 μM xanthine, 5 μM methyl viologen, 10 μM indigo carmine, and 10 μM renalase (with or without 30 mM β-NADP$^+$) in PBS buffer, pH 7.5, at 25 °C. Once the mixture was made anaerobic by 45 cycles of argon and vacuum (as above), xanthine oxidase (1–2 μM final) was added from a side arm to initiate the reduction of both the enzyme and dye. Spectra were recorded every 2 min until both species were fully reduced. The data were analyzed at wavelengths where the dye (610 nm) or the enzyme (470 nm) had absorption contributions that were spectrophotometrically independent of the other chromophore. At each of these wavelengths, the ratio of oxidized and reduced species was determined. The log of the ratio of the oxidized and reduced forms of indigo carmine was then plotted against the log of the same ratio for renalase, and the indigo carmine midpoint was substituted into the pH-corrected Nernst equation[29] to obtain the reduction potential for the renalase flavin cofactor.

The dissociation constant for the Ren$_{ox}$·β-NADP$^+$ complex was measured by monitoring the spectrophotometric perturbation of the flavin cofactor between 400 and 600 nm when β-NADP$^+$ was titrated to the oxidized enzyme. Renalase (20 μM) in PBS buffer at 25 °C was titrated with β-NADP$^+$ (0–8 mM). With each addition of β-NADP$^+$, the flavin absorption spectrum was measured. All spectra were corrected for dilution, and the fractional occupancy was assessed by the fraction of the extrapolated total perturbation observed at each β-NADP$^+$ concentration. This value was then used to obtain the concentration of unbound ligand. Plotting fractional occupancy versus unbound ligand gave a hyperbola that was fit to eq 1, where f is fractional occupancy, $K_{\beta\text{-NADP}^+}$ is the dissociation constant for the Ren$_{ox}$·β-NADP$^+$ complex and [β-NADP^+_f] is the concentration of unbound β-NADP$^+$.

$$f = [\beta\text{-NADP}^+_f]/(K_{\beta\text{-NADP}^+} + [\beta\text{-NADP}^+_f]) \quad (1)$$

Reductive Half-Reaction. The reduction of the renalase flavin cofactor was observed by mixing the anaerobic enzyme with anaerobic NADPH solutions at anomer equilibrium on a stopped flow spectrophotometer. Prior to the experiment, the instrument was made anaerobic by introducing a solution of glucose (20 mM) and glucose oxidase (15.5 U/mL) for approximately 16 h. Renalase (4 μM) in PBS buffer with 1 mM glucose was made anaerobic by placing it in a tonometer and exchanging the dissolved dioxygen for argon. Prior to sealing the vessel, ∼16 U of glucose oxidase was added to the side arm of the tonometer. The tonometer was then sealed and connected to an anaerobic manifold where 45 cycles of argon and mild vacuum were applied. Between each three exchanges, the solution was gently agitated to promote exchange of gases in the headspace of the vessel. After this procedure, the renalase/glucose solution was mixed with the glucose oxidase from the side arm and then mounted onto a Hi-Tech (now TgK) DX-2 stopped-flow spectrophotometer. This solution was then reacted with varied concentrations of α-NADPH (∼20–300 μM). Substrate solutions were prepared in PBS buffer with 1 mM glucose and sparged in an inverted glass syringe with purified argon for 10 min before 8 U of glucose oxidase was added and the syringe was mounted to the stopped-flow instrument. The two solutions were then mixed, and the bleaching of the flavin cofactor during reduction was observed at 458 nm. The data obtained was fit to a single exponential decay according to eq 2, where k_{obs} is the observed rate constant for reduction of the FAD cofactor, ΔA is the amplitude for the absorbance change, and C is the A_{458nm} end point.

$$A_{458nm} = \Delta A_1 \, e^{-k_{obs}t} + C \quad (2)$$

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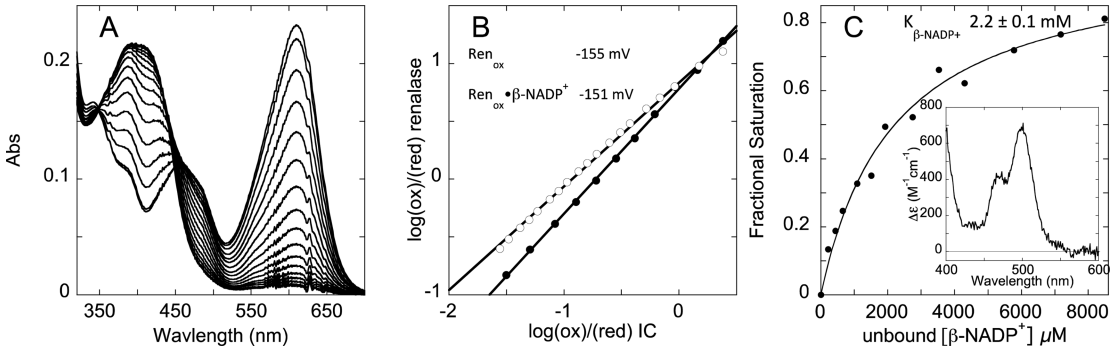

Figure 1. Redox states of renalase. (A) Simultaneous reduction of renalase (10 μM) and indigo carmine (10 μM) when reacted with 150 nM xanthine oxidase, 5 μM methyl viologen, and 300 μM xanthine in PBS buffer under anaerobic conditions. (B) Analysis of the data in panel A. The extent reduced for the enzyme and the dye was assessed where neither had a changing contribution from the other; 470 nm for renalase and 610 nm for indigo carmine. The data for Ren_{ox} are depicted in open circles and those for Renox·β-NADP$^+$ are shown as filled circles. (C) Measurement of the dissociation constant for the Ren_{ox}·β-NADP$^+$ complex. Renalase (20 μM) was titrated with β-NADP$^+$, and the perturbation of the flavin spectrum was observed. Inset depicts the ~80% saturation difference spectrum.

The dependence of the observed rate constant was then plotted against α-NADPH concentration and fit to eq 3 to determine the limit of the rate constant of reduction (k_{red}) and the dissociation constant for α-NADPH ($K_{\alpha\text{-NADPH}}$).

$$k_{obs} = k_{red}[\alpha\text{-NADPH}]/(K_{\alpha\text{-NADPH}} + [\alpha\text{-NADPH}]) \quad (3)$$

In order to observe potential NADP/FAD charge-transfer bands at longer wavelengths, the reductive process was also observed for 20 μM (final) renalase prepared in an equivalent manner and mixed with saturating α-NADPH (328 μM).

Oxidative Half-Reaction. The reoxidation of the renalase cofactor in the presence of dissolved dioxygen was observed in single turnover reactions by stopped-flow spectrophotometry. Renalase (14 μM) was prepared in tonometer in an equivalent manner to that described above. This solution was then mixed with an α/β-NADPH mixture of known ratio to supply sufficient α-NADPH to achieve ~50% reduction. The dissolved oxygen concentration in this solution was defined by sparging an inverted syringe containing the α/β-NADPH mixture with blended nitrogen and oxygen gases of known partial pressures supplied by a Maxtec maxblend gas mixer. The concentration of dissolved oxygen was confirmed by first sparging the reaction chamber of a Hansatech dioxygen electrode filled with PBS buffer with the blended gases to define the equilibrium concentration of dissolved dioxygen. Once the molecular oxygen concentration was established, the gas blend was applied to the α/β-NADPH mixture and sparged for 10 min before mounting the solution on the stopped-flow instrument. The solutions were then mixed and the reduction and ensuing reoxidation were observed at 458 nm. The data were fit to eq 4 in which k_{red} is the apparent rate constant for reduction and k_{ox} is the rate constant for reoxidation, ΔA_1 and ΔA_2 are the amplitudes for the two phases, and C is the end point absorbance.

$$A_{458nm} = \Delta A_1\, e^{-k_{red}t} + \Delta A_2\, e^{-k_{ox}t} + C \quad (4)$$

The influence of exogenous β-NADP$^+$ on the kinetics of the oxidative half-reaction was observed by reacting renalase (10 μM) with excess α-NADPH (20 μM) in the presence of 250 μM dioxygen and varied β-NADP$^+$ concentration (100−1600 μM). The reductive and oxidative processes were observed at 458 nm.

The Extent of Anomerization of an α/β-NADP$^+$ Mixture by Renalase. The capacity of renalase to catalyze anomerization independent of FAD cofactor reduction by α-NAD(P)H was assessed by incubation with an NADP$^+$ solution of known α/β ratio followed by high pressure liquid chromatography (HPLC). A 1/2.66 mixture of α/β-NADP$^+$ (275 μM total) was prepared by placing 40 mM β-NADPH in a solution of 10 mM sodium phosphate, pH 7.4, and allowing it to age at room temperature for 14 days. The inherently lower reduction potential of α-NADP and the anomer bias for the β-form ensures that similar amounts of the α- and β-NADP$^+$ anomers accumulate.[30] The oxidized forms were separated from the reduced and other contaminants by loading the aged sample onto a 20 cm × 12.5 mm Q-sepharose column and eluting with a linear gradient of NaCl from 0 to 150 mM. The α/β-NADP$^+$ mixture was then diluted to 75 μM, and 50 μL was chromatographed using a 4.6 mm × 150 mm Xterra C18 reverse phase column coupled to a Waters 600 E HPLC pump and a Waters 2487 dual wavelength detector. The α- and β-anomer mixture was then separated using an isocratic mobile phase of 10 mM sodium phosphate, pH 7.5. Renalase (5.6 μM final) was then added to the α/β-NADP$^+$ solution, and 50 μL volumes were withdrawn periodically, filtered through a 0.5 mL Amicon 10 kDa centrifugal filter, and chromatographed as above.

The propensity of the reduced enzyme to catalyze anomerization of NAD(P)$^+$ was assessed using similar methods but with prior reduction of renalase. The enzyme was reduced using an adaptation of the methods used to reduce the chromophores in the reduction potential measurements described above. The reaction mixture was placed in an anaerobic cuvette and consisted of 300 μM xanthine, 2 μM methyl viologen, and 6.0 μM renalase in PBS buffer, pH 7.5, at 25 °C. The mixture was made anaerobic by 45 cycles of argon and vacuum (as above), and then xanthine oxidase (1−2 μM final) was added from a side arm to initiate the reduction of the enzyme. The extent of reduction was assessed spectrophotometrically using a Hewlett-Packard 8453 spectrophotometer. A 1/2.66 mixture of α/β-NADP$^+$, 75 μM (final), was then added from a second side arm, and the sample was allowed to incubate for 60 min before the protein components were removed by filtration through a 10 kDa nominal molecular weight centrifugal filter. The chromatograms obtained were then compared with two controls, the first that included all

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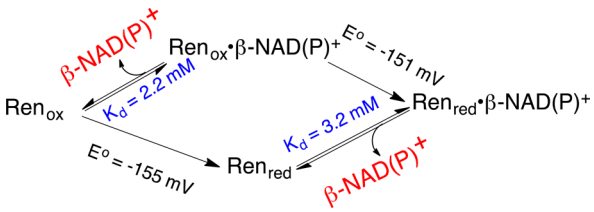
reaction components minus the mixture of α/β-NADP$^+$ and the second that included only the α/β-NADP$^+$ mixture.

RESULTS

Redox-Linked Measurement of β-NADP$^+$ Binding Affinities. The renalase reduction potential was determined using a spectrophotometric method that compares the extent of reduction of the enzyme relative to a dye of known potential when electrons are supplied from xanthine oxidase reacting with xanthine under anaerobic conditions.[28] In this experiment, the dye serves a similar function to that of a reference electrode. Figure 1A includes spectra obtained as renalase and indigo carmine are reduced by the xanthine/xanthine oxidase system. Figure 1B shows the midpoint for the dye compared with the extent of reduction of renalase. This plot allowed a reduction potential for renalase of −155 mV to be calculated. The experiment was repeated with saturating β-NADP$^+$ (*vide infra*) to obtain a reduction potential for the Ren$_{ox}$·β-NADP$^+$ complex of −151 mV. No flavin semiquinone is observed to accumulate during reduction suggesting that the second one-electron potential is higher than the first. The reduction potential for α-NAD(P)H is −340 mV[30] indicating that the redox driving force (excluding the contribution of anomer equilibria) for the reduction of the flavin is −35.6 kJ/mol. Coupling this to the reduction of dioxygen yields −122.5 kJ/mol driving force for the complete catalytic cycle.

The dissociation constant for the renalase product complex (Ren$_{ox}$·β-NADP$^+$) was measured using perturbation of the flavin absorption spectrum in the presence of the ligand (Figure 1C). These data indicate a relatively weak complex with a dissociation constant of 2.2 mM and are consistent with the prior work of Milani et al. who measured a value of 1.6 mM under similar conditions.[10] The reduction potentials of Ren$_{ox}$ and the Ren$_{ox}$·β-NADP$^+$ complex and the dissociation constant for the Ren$_{ox}$·β-NADP$^+$ complex complete three sides of a thermodynamic box linking enzyme reduction and β-NADP$^+$ complexation. The small difference in reduction potentials for Ren$_{ox}$ and Ren$_{ox}$·βNADP$^+$ indicate that the dissociation constants of the oxidized or reduced renalase β-NADP$^+$ complexes are similar (Scheme 2). These data suggest that in the Ren$_{ox}$·β-NADP$^+$ complex the nicotinamide ring and the isoalloxazine ring of the renalase FAD cofactor are not in sufficient proximity to influence the cofactor's reduction potential.

Scheme 2



Reductive Half-Reaction. The reductive half-reaction of renalase was observed independent of oxidative chemistry by combining the enzyme with α-NADPH and excluding dioxygen (Figure 2). The α-NADPH substrate was supplied from a solution of NADPH at anomeric equilibrium (1.5% α-anomer). Under these conditions, the α-NADPH substrate initiates reduction of the flavin observed as a decrease in the absorption transitions centered around 458 nm (Figure 2A,B). Freshly dissolved β-NADPH does not induce flavin reduction (Figure 2B). The dependence of the observed rate constant for reduction of the renalase flavin cofactor on the α-NADPH concentration could be fit to a rectangular hyperbola according to eq 2 (Figure 2C). This equation assumes rapid equilibrium binding that diminishes the observed rate constant of reduction at subsaturating substrate concentrations.[31] The asymptote is thus the limit of the rate constant for reduction (k_{red} = 40.2 ± 1.3 s^{-1}) and the concentration at which the observed rate constant for reduction is half maximal is the dissociation constant for α-NADPH ($K_{\alpha\text{-NADPH}}$ = 19.9 ± 2.6 μM). These experiments required the use of low renalase concentration (2 μM) in order to be pseudo-first-order with a range of α-NADPH concentrations that reasonably span the α-NADPH binding isotherm according to the $K_{\alpha\text{-NADPH}}$ value. A second experiment was also undertaken with relatively high renalase concentration (20 μM) and α-NADPH (328 μM) concentrations in order to observe potential charge-transfer absorption transitions at longer wavelengths that would indicate proximity of the nicotinamide and FAD isoalloxazine rings (Figure 2A,B).[32,33] Figure 2B includes a long wavelength trace that indicates no significant accumulation of charge-transfer transitions before, during, or after reduction. Figure 2A includes deconvoluted component spectra obtained from single exponential fits to photodiode array data for the reductive half-reaction. These spectra also show that no significant new absorbance transitions indicative of charge-transfer absorption transitions were observed. It is therefore conceivable that neither α-NAD(P)H nor β-NAD(P)$^+$ nicotinamide rings stack with the flavin isoalloxazine ring (*vide infra*). The first spectrum acquired at 1.54 ms indicates that the flavin absorption transitions become resolved directly prior to reduction (Figure 2A, solid line). This perturbation may arise either in the Ren$_{ox}$·α-NADPH complex or potentially in a ribose ring-open iminium ion complex that forms rapidly prior to flavin reduction such as proposed in the hypothetical catalytic cycle depicted in Scheme 3.

Oxidative Half-Reaction. The oxidative half-reaction of renalase is observed as a monophasic return to the oxidized state of the flavin cofactor. Figure 3A summarizes the data obtained when renalase was reduced by limiting concentrations of α-NADPH in the absence or presence of pseudo-first-order concentrations of dioxygen. The reductive and oxidative processes differ sufficiently to allow both phases to be observed. When dioxygen concentration was titrated in successive reactions, the observed rate constant for reoxidation had a linear dependence on the molecular oxygen concentration that passed through the origin (Figure 3B). This indicates that the reaction of reduced renalase with dioxygen is reliant on collision without prior complexation. The slope of the dependence indicated a rate constant of 2.95 × 10^3 ± 70 M^{-1} s^{-1}, a value similar to that of autoxidation of FAD in solution[34] suggesting that renalase does not promote the reaction of its cofactor with dioxygen and dictating that reoxidation will generally be the rate-limiting process *in vitro* under conditions of atmospheric dioxygen (~250 μM).

In order to evaluate the timing of the release of the β-NAD(P)$^+$ product with respect to reoxidation of the flavin cofactor, exogenous β-NAD(P)$^+$ was titrated in single turnover reactions (Figure 3C). These data indicated that increasing concentrations of the β-nicotinamide product had no influence on the observed rate constant for reoxidation suggesting that reoxidation is not contingent on the release of β-NAD(P)$^+$.

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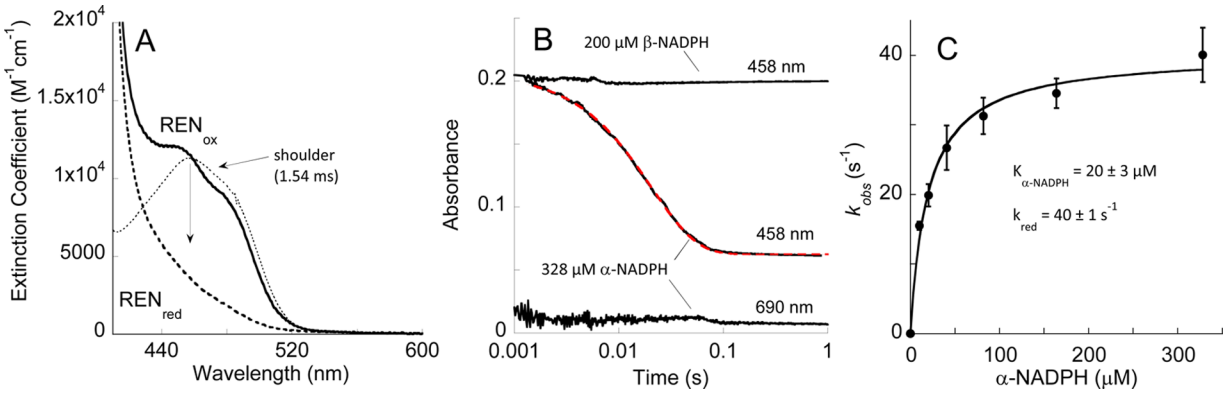
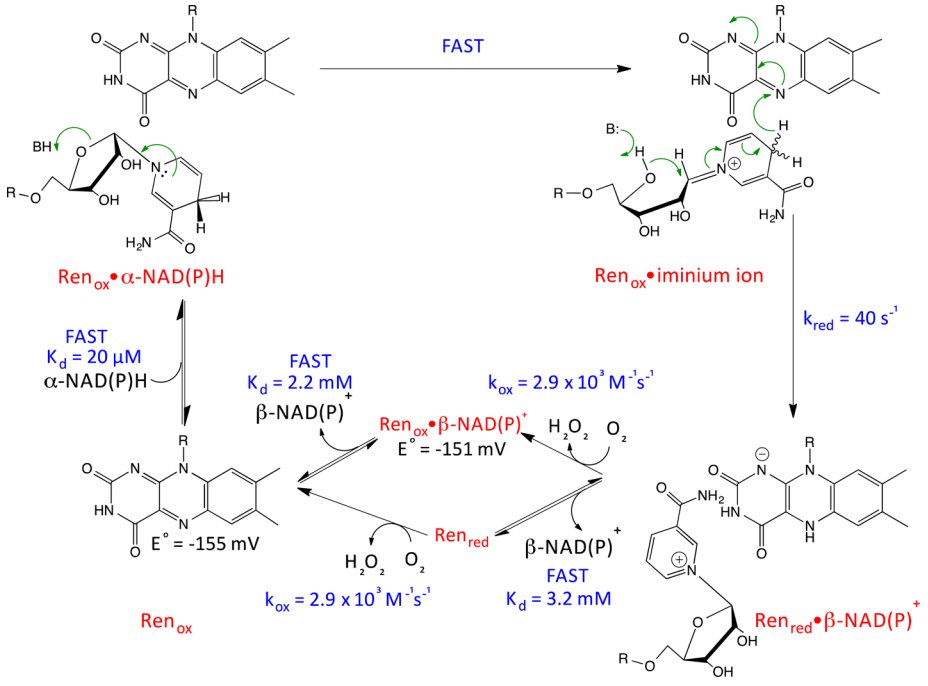


Figure 2. Reductive half-reaction of renalase. (A) Initial (solid line) and final (dashed line) absorption spectra when 328 μM α-NADPH was reacted with 20 μM renalase under anaerobic conditions. The dotted line spectrum is the spectrum of resting oxidized renalase. (B) Absorbance traces at 458 and 690 nm when 328 μM α-NADPH was reacted with 20 μM renalase under anaerobic conditions. The dashed line is the fit (red) to a single exponential phase (eq 2) with a rate constant of 41 s^{-1}. Upper trace is for the reaction of 20 μM renalase with 200 μM solution of freshly dissolved β-NADPH under anaerobic conditions. (C) Dependence of the observed rate constant for reduction when varied pseudo-first-order concentrations of α-NADPH were reacted with 2 μM renalase under anaerobic conditions. The fit is to eq 3 and gave a limiting rate constant for reduction of 40 $\pm$ 1 s^{-1} and a dissociation constant for α-NADPH of 19.8 $\pm$ 2.6 μM.

Scheme 3



Consistent with the observed complexation of β-$NADP^+$ with the oxidized enzyme, our data show that this molecule is weakly competitive with α-NADPH since the observed rate of reduction slows with increasing β-$NADP^+$ concentration (observed as a decrease in the amplitude for the reductive phase). Therefore, we conclude that dioxygen and the nicotinamide ring of β-$NAD(P)^+$ do not compete for access to the reduced flavin cofactor during reoxidation. Overall the data are consistent with a kinetically ordered release of products with β-$NAD(P)^+$ dissociating prior the reoxidation of the enzyme. Exogenous β-$NAD(P)^+$ can populate the Ren_{ox}·β-$NAD(P)^+$ complex and hinder α-NADPH association to the oxidized enzyme; however, the physiological relevance of this observation is negligible because the concentration of β-$NAD(P)^+$ in the blood is low relative to its dissociation constant.[35]

Extent of Anomerization of α/β-$NADP^+$ by Oxidized Renalase. The ability of oxidized and reduced renalase to catalyze non-redox-coupled anomerization was assessed by incubating oxidized or reduced renalase with an α/β-$NADP^+$ mixture of known anomer ratio. The ratio of both anomers was remeasured after incubations with oxidized or reduced renalase for defined times over an hour. Figure 4 illustrates that for renalase in either oxidation state the ratio of α- to β-$NADP^+$ is unchanged. This establishes that anomerization does not occur before FAD cofactor reduction, while it is reduced, or after its reoxidation and suggests that the oxidative and epimerization activities of renalase are mechanistically linked via a common intermediate.

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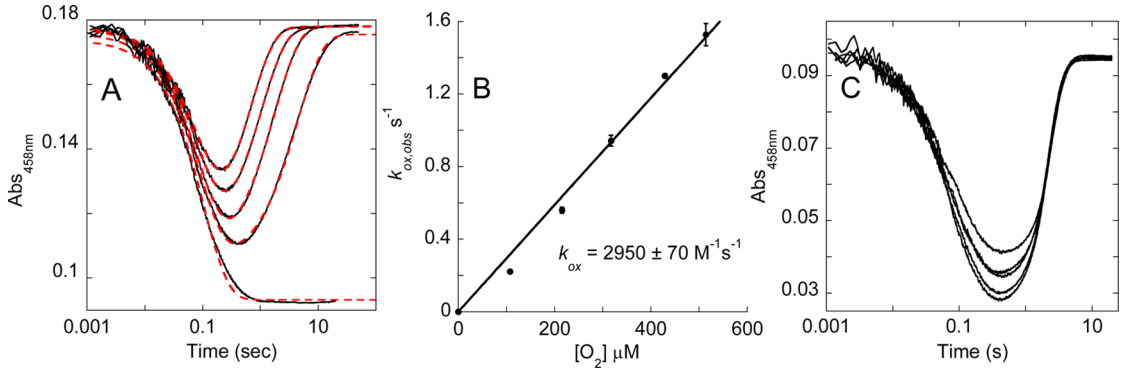

Figure 3. Single turnover of renalase with limiting α-NADPH. (A) Traces observed at 458 nm when 14 μM renalase was reacted with 5.7 μM α-NADPH in varied pseudo-first-order concentrations of molecular oxygen. The traces shown in ascending order are for 0, 107, 215, 317, and 514 μM dioxygen. Data obtained without dioxygen were fit to a single exponetial decay (eq 2), and data obatined with dioxygen were fit to two exponential phases (eq 4) (fits shown as dashed red lines). (B) The dependence of the observed rate constant for reoxidation (k_2) on the concentration of molecular oxygen fit to a straight line that passes through the origin. The slope of the line indicates a second-order rate constant for the addition of dioxygen of 2.9×10^3 M^{-1} s^{-1}. (C) Influence of β-NADP$^+$ on single turnover kinetics. Renalase (10 μM) was mixed with α-NADPH (20 μM) and varied β-NADP$^+$ (0−1600 μM) in the presence of 250 μM dioxygen. The traces in ascending order are for 100, 200, 400, 800, and 1600 μM β-NADP$^+$.

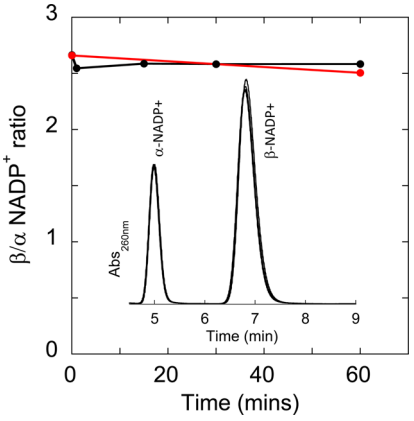

Figure 4. Extent of anomerization by renalase in the oxidized and reduced states. Data depict the extent of anomerization of a 75 μM mixture of 1/2.66 α/β-NADP$^+$ in the presence of 5.6 μM oxidized (black line) and 6.0 μM reduced (red line) renalase. The observed α/β NADP$^+$ ratio was based on HPLC peak area integration. The inset shows HPLC data for the oxidized enzyme experiment (four overlaid chromatograms are shown). Species were separated using a Xterra reverse phase C18 column running isocratically in 10 mM sodium phosphate buffer, pH 7.5.

DISCUSSION

Renalase is a recently discovered flavoprotein that has been widely reported to be a kidney hormone whose endocrine function is to lower circulating concentrations of catecholamine neurotransmitters, such as epinephrine, thereby lowering blood pressure and heart rate.[2] Reported slow rates of turnover with catecholamine substrates *in vitro* do not address catalytic enhancements, nor do they offer a definitive demonstration of the products formed or the reaction stoichiometry.[16,17] As such the claimed "monoamine oxidase C" activity of renalase continues to be challenged in the literature.[21,22,36−38] The identification of α-NAD(P)H oxidase/anomerase activity for renalase calls for re-evaluation of many of the reported physiological observations because the only known vasoactive molecule to be consumed or liberated by this activity is H_2O_2.[24,25,38] However, the concentration of this product is contingent on the low equilibrium concentration of α-NADPH that would be maintained near zero by renalase activity, meaning that the rate of H_2O_2 production by renalase would be dependent on the *in vivo* β- to α-NAD(P)H anomerization rate constant (ca. 10^{-6} s^{-1}).[26]

Reconciling each of the observations and conclusions made for renalase is complicated by the use of different preparations of the enzyme. Renalase was initially expressed as a recombinant glutathione synthase fusion and isolated using glutathione sepharose.[2] Later preparations were isolated from urine using an antirenalase affinity column.[16] More recent preparations have used heterologous expression in *Escherichia coli* from a synthetic codon-optimized renalase gene. Heterologous expression from the optimized gene yields a modest fraction of folded enzyme and a large fraction of inclusion body peptide.[7] The smaller, as expressed, soluble fraction can be purified by conventional methods[10,23,39] and was the form used to obtain the available X-ray crystal structure.[10] It is likely that this is the native form of renalase because it is this form that exhibits the α-NAD(P)H oxidase/anomerase activity described herein.[23] The second reported preparation method involves solubilizing inclusion body peptide and refolding by dilution and pH titration.[7] This form of renalase does not have the spectrophotometric properties of the soluble fraction and does not exhibit α-NAD(P)H oxidase/anomerase activity[23] but has been used in a number of physiological studies of renalase that report vasoactivity.[7,17]

We have completed a preliminary examination of the catalytic cycle of the as expressed soluble renalase reacting with α-NADPH. We define substrate affinities, the rate constants for those steps that contribute to the turnover number and propose a hypothetical mechanism for the observed activity. Scheme 3 depicts an annotated chemical mechanism that accounts for each of the observations made in this study. The data suggest that the redox and anomerization activities of renalase are coupled via a shared intermediate because neither the oxidized nor the reduced enzyme can catalyze anomerization of NADP$^+$ (Figure 4). Catalysis commences with the association of α-NAD(P)H to renalase to form the Ren_{ox}·α-NAD(P)H complex ($K_d \approx 20$ μM). The lack of charge-transfer absorbance transitions for this complex suggests that the nicotinamide base does not stack with the

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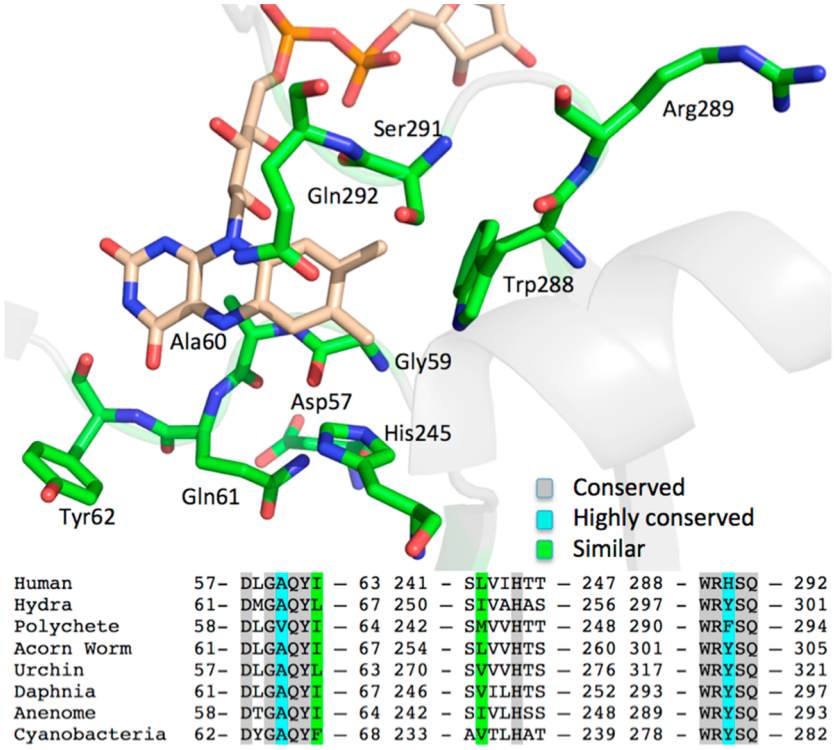

Figure 5. Conservation within the renalase active site. Structure shown is for human renalase (PDB ID 3QJ4). Sequence data are for the primary structures of renalase and unconfirmed renalase-like proteins from a number of divergent phyla.

flavin isoalloxazine (Figure 2).[40] It is proposed instead that rapid delocalization of the dihydropyridine lone pair and protonation of the bridging oxygen atom of the ribose ring forms an iminium ion intermediate. The iminium ion then recyclizes at the ribose, simultaneously causing the formation the β-NAD(P)$^+$ product with concerted reduction of the flavin cofactor ($k_{red} \approx 40\ s^{-1}$). Much like the Ren$_{ox}$·$\alpha$-NAD(P)H complex, the nicotinamide ring of the Ren$_{red}$·β-NAD(P)$^+$ complex is oriented with respect to the flavin isoalloxazine ring system such that it does not promote charge-transfer transitions (Figures 1C and 2B). In this position, the β-nicotinamide of the product complex does not impede the reaction of the reduced flavin with dioxygen ($k_{ox} \approx 2.9 \times 10^3\ M^{-1}\ s^{-1}$) so that the β-NAD(P)$^+$ release and reoxidation steps occur independently (Figure 3C). Further evidence for this is that the reduction potentials of the unliganded oxidized enzyme and the Ren$_{ox}$·β-NAD(P)$^+$ complex are ostensibly the same. This also indicates that the dissociation constant of the Ren$_{ox}$·β-NAD(P)$^+$ complex ($K_d \approx 2.2$ mM) is similar to the Ren$_{red}$·β-NAD(P)$^+$ complex (Figure 1, Schemes 2 and 3). In addition, the lack of influence of β-NAD(P)$^+$ on the observed rate constant for reoxidation indicates that the rate constant for reoxidation ($k_{ox} \approx 2.9 \times 10^3\ M^{-1}\ s^{-1}$) is the same for the unliganded reduced enzyme and the Ren$_{red}$·β-NAD(P)$^+$ complex (Figure 3C). A weak reassociation propensity of β-NADP$^+$ with oxidized renalase forms a dead-end complex that can retard binding of α-NAD(P)H (Figure 3C) suggesting that the nicotinamide substrate and product occupy the same or overlapping binding pockets.

Renalase turnover has two rate contributing chemistries, two-electron reduction of the flavin ($\sim 40\ s^{-1}$) and its subsequent reoxidation ($2.9 \times 10^3\ M^{-1}\ s^{-1}$). The latter of these will typically have the greatest contribution to the turnover number *in vitro* under conditions of atmospheric oxygen ($\sim 250\ \mu M$ O_2). However, in blood the concentration of available α-NAD(P)H will be negligible as a consequence of the large driving force for the renalase reaction ($\Delta G \approx 120$ kJ/mol),[26] while dissolved dioxygen will be approximately constant at $\sim 140\ \mu M$. Under these conditions, renalase activity will be primarily responsive to the α-NAD(P)H concentration, consistent with a role for renalase of minimizing the α/β-NAD(P) ratio.

Renalase is reported to be expressed in numerous tissues,[2,9] and the conserved active site residue motif can be identified in homologous proteins from animals such as anemones, polycheates, daphnia, and even single-celled organisms such as cyanobacteria (Figure 5). This implies that the root physiological role of renalase is more pervasive than vasodilation. The α-NAD(P)H oxidase/anomerase activity of renalase is significantly exothermic, which implies that the purpose of this activity is to use the considerable oxidative power of dioxygen to deplete the α-NAD(P)H concentration. In the absence of renalase, the inherently lower reduction potential of α-NAD(P)H molecules (-340 mV) dictates that they will tend to oxidize by reducing β-NAD(P)$^+$ or other singlet molecules of higher potential and become isolated, unable to be reincorporated into metabolism. This would result in a steady but inexorable loss of function for a major fraction of available NAD(P) molecules. It is therefore reasonable to conclude that the renalase α-NAD(P)H oxidase/anomerase activity is important to all metabolism in order to maintain the α/β-NAD(P)H ratio at ostensibly zero such that the preponderance of nicotinamide dinucleotides remain available for redox cycling.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

FAD, flavin adenine dinucleotide; MAO, monoamine oxidase; NADP, nicotinamide adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide; HPLC, high performance liquid chromatography

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