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Renalase Is an α-NAD(P)H Oxidase/Anomerase

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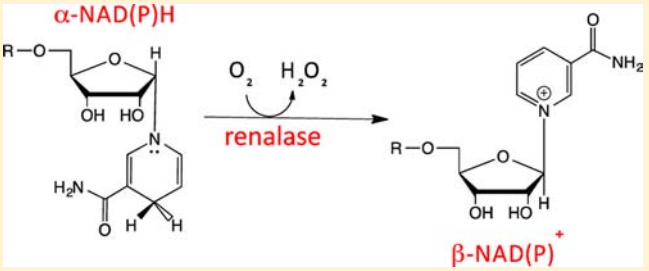
ABSTRACT: Renalase is a protein hormone secreted into the blood by the kidney that is reported to lower blood pressure and slow heart rate. Since its discovery in 2005, renalase has been the subject of conjecture pertaining to its catalytic function. While it has been widely reported that renalase is the third monoamine oxidase (monoamine oxidase C) that oxidizes circulating catecholamines such as epinephrine, there has been no convincing demonstration of this catalysis *in vitro*. Renalase is a flavoprotein whose structural topology is similar to known oxidases, lysine demethylases, and monooxygenases, but its active site bears no resemblance to that of any known flavoprotein. We have identified the catalytic activity of renalase as an α-NAD(P)H oxidase/anomerase, whereby low equilibrium concentrations of the α-anomer of NADPH and NADH initiate rapid reduction of the renalase flavin cofactor. The reduced cofactor then reacts with dioxygen to form hydrogen peroxide and releases nicotinamide dinucleotide product in the β-form. These processes yield an apparent turnover number (0.5 s^{-1} in atmospheric dioxygen) that is at least 2 orders of magnitude more rapid than any reported activity with catechol neurotransmitters. This highly novel activity is the first demonstration of a role for naturally occurring α-NAD(P)H anomers in mammalian physiology and the first report of a flavoprotein catalyzing an epimerization reaction.



INTRODUCTION

Human renalase was first discovered in 2005 by Xu et al.[1,2] and its discovery spurred considerable interest because the protein was reported to be secreted into the blood by the kidney as a hormone to down-modulate blood pressure and cardiac output.[3] These observations have since been associated with the high incidence of cardiovascular disease in patients with end-stage renal disease,[4] emphasizing that diminishment of the endocrine function of the kidney is wholly detrimental to cardiac function.[1,5,6] In addition, mice deficient in renalase have been reported to exhibit elevated blood pressure[4] and human renalase polymorphisms have been linked with an increased incidence of diabetes and stroke.[7,8] Since its discovery, a definitive demonstration of the catalytic function of renalase has remained elusive. While it has been widely reported that renalase lowers blood pressure and modifies heartbeat by catabolizing circulating catecholamines,[9−15] neither the specific substrate(s) and product(s) nor the overall stoichiometry for the reaction has been convincingly established. *In vivo* studies in rodents show that the concentration of circulating catecholic neurotransmitters is lowered by intravenous injection of renalase.[1,4,14] However, *in vitro* studies have failed to demonstrate significantly catalytic consumption of catecholic neurotransmitters (or their precursors).[14,16] It has been claimed that renalase mobilizes four electrons by acquiring two electrons from NAD(P)H in order to catalyze a net two-electron oxidation and cyclization of epinephrine to form adrenochrome.[15] Such a transformation for epinephrine is, however, facile in the absence of an enzyme catalyst and readily observed in oxygenated solutions under a variety of conditions.[17,18] Similarly, the observation of apparent slow oxidation of reduced pyridine nucleotide cofactors in the presence of renalase is complicated by the inherent instability of these molecules at pH values near or below neutrality.[19] As a consequence, claims of renalase directly catalyzing the breakdown of catecholamines has been challenged in both letters and research articles.[16,20,21]

The discord in regard to the activity of renalase has developed both from its reported physiological function and from its structural similarity to known redox enzymes. The primary structure of the protein has at least four forms that result from RNA splicing patterns for seven exons. Only the two longest sequences that include protein derived from at least six exons show direct similarity to known flavoproteins.[9,21] The three-dimensional structure of renalase isoform 1 (342 amino acids) was published by Milani et al. in 2011.[22] This structure indicated a fold consistent with a large structural family of flavoproteins that include oxidases, lysine demethylases, and monooxygenases.[23−27] The apparent active site of renalase was observed to be a clearly delineated pocket in the surface of the protein that provides access to the *si*-face of a noncovalently bound flavin adenine dinucleotide (FAD) cofactor. Within this cavity, the constellation of residues that are conserved in renalase primary structures gave no clear indication for any known activity of a flavoprotein. However, renalase was observed to readily form an adduct with sulfite ions at the

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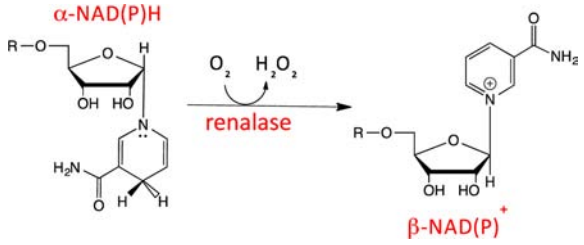
FAD isoalloxazine N5 position, a curious characteristic that is most commonly associated with oxidase activity.[22,28,29]

In this article, we identify renalase as an α-NAD(P)H oxidase/anomerase whereby the substrate α-dihydropyridyl ring is oxidized by transferring two electrons to the flavin cofactor and the configuration of the ribose C1 is converted from α to β. The reduced FAD cofactor then reoxidizes by reacting with dioxygen to yield hydrogen peroxide (Scheme 1). This highly novel transformation is both unprecedented catalytic chemistry and the first physiological link to α-pyridine nucleotides in higher organisms.

Scheme 1



MATERIALS AND METHODS

Materials. Plasmids were purified using the Qiagen Midi-Prep plasmid preparation kit. Oligonucleotides were synthesized by Operon. Vector pET28a(+) was obtained from Novagen. Restriction enzymes, DNA modification enzymes, and competent (DH5α and BL21 (DE3)) *Escherichia coli* were obtained from New England Biolabs. FAD, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), potassium phosphate, isopropyl-β-thiogalactopyranoside (IPTG), and sodium chloride were from ACROS. Electrophoretic grade agarose was obtained from ICN Biomedicals. Luria–Bertani (LB) broth (Lennox) powder was from Fisher Scientific. Kanamycin and β-NADPH were purchased from Alexis. β-NADP$^+$ was purchased from Calbiochem. β-NADH, β-NAD$^+$, and epinephrine were from Sigma-Aldrich.

Cloning and Expression of Human Renalase. The gene encoding full-length WT human renalase (isoform 1) optimized for expression in *E. coli* and incorporating *Nde*I and *Xho*I sites at the 5′ and 3′ ends was purchased from Enzymax. The gene was supplied in plasmid pUC57 and subcloned into the *Nde*I and *Xho*I restriction sites of pET-28a(+) expression vector (Novagen). The *Nde*I insertion of pET-28a(+) plasmid incorporates an N-terminal His-Tag fusion. This plasmid (pHSRENHT) was transformed into chemically competent *E. coli* BL21 (DE3) cells (Novagen). All media used with transformed cells included 100 μg/mL kanamycin. Transformants were plated onto LB agar, and a single isolated *E. coli* colony was cultured in LB broth. Individual 1 mL cell stocks were made by adding sterile glycerol to a final concentration of 20% to cells grown to early log phase, and these were then stored at −80 °C.

Renalase Expression and Purification. For expression, 1 mL cell stocks were thawed, plated onto LB agar with 100 μg/mL kanamycin, and grown overnight at 37 °C (50 μL/plate). The lawn of cells obtained was then transferred to LB broth (two plates per liter of broth) and grown at 37 °C in a shaking incubator (220 rpm) to midlog phase (OD_{600nm} = 0.5) and the temperature was lowered to 22 °C. The culture was then grown to $OD_{600nm} \approx 1.0$ (~1 h) and induced with 0.1 mM IPTG and left to express renalase for 20 h before the cells were harvested by centrifugation (4000*g* for 30 min). Unless otherwise stated, all subsequent purification steps were performed at 4 °C. Cell pellets were resuspended in 20 mM HEPES buffer, pH 7.5 (approximately 10 mL/L culture), and lysed by sonication using a Branson 450 sonicator (3 × 240 s at 50 W). The temperature of the cell suspension was maintained below 10 °C by immersing the sample vessel in a slurry of ice and water. Lysed cells were centrifuged at 32800*g* for 30 min, the pellet was discarded, and the supernatant was loaded onto a 12.5 × 150 mm^2 Co^{2+} Talon column (BD Biosciences) equilibrated with 20 mM HEPES buffer, pH 7.5. Protein was eluted with a 1 mL/min two-step protocol. Initially contaminating proteins were eluted with 150 mL of 10 mM imidazole, 50 mM HEPES buffer, pH 7.5, then a gradient from 10 mM to 150 mM imidazole in the same buffer was used to elute renalase. Distinctly yellow fractions were pooled. Imidazole was removed, and the buffer was exchanged to PBS (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) by repeated concentration and dilution using 10 kDa nominal molecular weight cutoff centrifugal concentrator (Millipore, Amicon) to achieve a net ≥1000-fold exchange. Aliquots of purified concentrated renalase were then stored at −80 °C.

Quantitation. The concentrations of NADPH and NADH solutions were determined using the published 340 nm extinction coefficient of 6200 M^{-1} cm^{-1}.[30] The extinction coefficient for renalase was determined by liberating the FAD cofactor from the protein. Initially, the absorption spectrum of 9 μM renalase was recorded using a Hewlett-Packard 8453 spectrophotometer. SDS to 1% final concentration was then added to denature the protein and release the flavin, and the spectrum was again recorded. The spectrum of the unbound flavin was then corrected for dilution and the known extinction coefficient for this molecule at 450 nm (ε_{450nm} = 11300 M^{-1} cm^{-1}) was used to calculate the extinction coefficient of renalase (ε_{458nm} = 11330 M^{-1} cm^{-1}). Epinephrine was quantified by weight.

Substrate/Product Analysis. The apparent substrate for renalase was detected as a contaminant of β-NAD(P)H stocks that caused rapid reduction of the renalase flavin cofactor. The proportion of the substrate in NAD(P)H solutions was determined by two methods. First, relatively high concentrations of β-NADPH were mixed with renalase using a Hi-Tech (now TgK) stopped-flow spectrophotometer under anaerobic conditions. Prior to the experiment, the instrument was scrubbed of residual dioxygen for ~16 h using a solution of 50 mM glucose and 15.5 U/mL glucose oxidase. Renalase in PBS buffer was prepared in a tonometer by adding 8 μM enzyme with 1 mM glucose in the body of the vessel and 16 U of glucose oxidase in a side arm (U is defined as 1 unit of enzymatic activity or 1 μmol/min). The tonometer was then subject to 45 cycles of low vacuum followed by argon gas with mild agitation to exchange all dissolved dioxygen. Once anaerobic, the renalase/glucose solution was mixed with the glucose oxidase from the tonometer side arm, and the vessel was mounted onto the stopped-flow instrument. NADPH solutions were prepared with 1 mM glucose and made anaerobic by sparging with argon for 5 min and then adding in 8 U of glucose oxidase immediately prior to mounting onto the instrument. Renalase and NADPH were then mixed and the extent of flavin cofactor reduction was recorded at 458 nm. The second method used to determine the proportion of substrate in NAD(P)H solutions was to observe the change in molecular oxygen concentration when high concentrations of NAD(P)H were added to low micromolar concentrations of renalase in PBS buffer using a Hansetech dioxgen electrode. Assuming 1:1 substrate to dioxygen reaction stoichiometry (*vide infra*), the concentration of substrate could be determined from the amplitude of the observed change in molecular oxygen concentration.

Evidence for the identity of the substrate was obtained from high-pressure liquid chromatography (HPLC) analysis. NAD(P)H solutions (10 mM) were diluted by a factor of 50 and filtered using a Millipore 0.5 mL 10 kDa nominal molecular weight centrifugal filter device. This solution (50 μL) was then injected onto a Waters Xterra C18 cartridge column (4.6 × 150 mm^2) run isocratically at 0.5 mL/min with 10 mM NaPi, pH 7.5, coupled to a waters 600E pump and Waters 2487 detector. The elution of components was observed at both 260 and 340 nm. The dominant components having absorbance at 340 nm were collected, and their spectra were recorded. A second sample was then prepared in an equivalent manner but with the addition of renalase to 5 μM. After incubation for 2 min, this sample was filtered to remove the enzyme and subject to HPLC analysis.

HPLC identification of the product of the renalase reaction was based on coelution. Chromatography conditions were as described above. Initially, a 50 μL control sample from a solution of 340 μM

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NADH in PBS buffer was separated into its α and β-NADH components. A second 50 μL sample was then reacted with 5 μM renalase for 2 min and filtered using a Millipore 0.5 mL 10 kDa nominal molecular weight centrifugal filter device before being chromatographed in an equivalent manner. A third 340 μM sample was then prepared with the addition of 15 μM β-NAD^+ and chromatographed. The retention time of the product of the renalase reaction was compared with that of the sample spiked with β-NAD^+ to identify the product.

The nicotinamide product of a renalase/NADPH reaction was identified as β-$NAD(P)^+$ by nuclear magnetic resonance (NMR). A 40 mM solution of NADPH was prepared in 10 mM NaPi buffer, pH 7.4, in deuterium oxide solvent. The ^{1}H NMR spectrum was then recorded by collecting and transforming 256 free induction decays (FIDs) soon after preparing the sample on a Bruker 300 MHz NMR instrument. Renalase (10 μM, 0.3 U) and catalase (0.2 U) were then added to the NMR tube and mixed by repeated pipetting with a Pasteur pipet. Further spectra were then recorded at specific times over the next 100 min. A control NADPH sample was then prepared at the same concentration in the same buffer and monitored similarly. Prior to each spectrum (sample and control) the contents of the NMR tube were re-equilibrated with atmospheric molecular oxygen by repeatedly drawing the solution into a Pasteur pipet. The concentration of the product was approximated from the relative integrations of known resonances for β-NADPH and the singlet resonance for proton N2 (2 position of the nicotinamide) of the species observed to accumulate in the reaction. These spectra were compared with the proton spectra of 1 mM β-$NADP^+$ alone and 1 mM β-$NADP^+$ in the presence of 40 mM β-NADPH.

Evaluation of Epinephrine as a Substrate for Renalase. Epinephrine was tested as a substrate for renalase using four approaches. In the first, the reported accumulation of adrenochrome in the presence of renalase was evaluated. The concentration dependence of the formation of adrenochrome was observed by adding varied concentrations of epinephrine (0−400 μM, prepared in 10 mM HCl) to a solution of PBS buffer and then observing the increase in absorbance at 480 nm at 25 °C. The initial rate at each epinephrine concentration was measured by fitting the first 200 s to a straight line and dividing the slope by the extinction coefficient for adrenochrome (4020 M^{-1} cm^{-1}).[18] To determine whether renalase or NADPH accelerates this process, 10 μM renalase and then 10 μM renalase with freshly prepared β-NADPH to 400 μM was added to 400 μM epinephrine (final), and the initial rate was recorded.

In the second approach, the effect of epinephrine on the observed single-turnover kinetics was assessed by stopped-flow spectrophotometry. Non-pseudo-first-order single-turnover reactions were initiated by mixing a limiting concentration of the α-NADPH (~6 μM in 400 μM β-NADPH) in PBS buffer equilibrated with atmospheric dioxygen (~250 μM) with 11.4 μM aerobic renalase in the same buffer. The reaction was monitored by observing the reduction and reoxidation of the enzyme's flavin cofactor at 458 nm. Epinephrine (400 μM) was then added to the α-NADPH solution, and the observation was repeated. In order to evaluate whether some soluble component of blood modulated or reacted with renalase, this observation was repeated with the α-NADPH/epinephrine solution prepared in fresh cell-free blood plasma. Solely for comparison, the data were fit to eq 1, which describes three successive first-order events. In this equation, k_1 is the apparent rate constant for reduction, k_2 is an additional unassigned phase, k_3 is the apparent rate constant for reoxidation, ΔA_{1-3} are the respective absorbance amplitudes for the phases observed, and C is the end point absorbance.

$$A_{458\text{nm}} = \Delta A_1\, e^{-k_1 t} + \Delta A_2\, e^{-k_2 t} + \Delta A_3\, e^{-k_3 t} + C \qquad (1)$$

Isothermal titration calorimetry (ITC) was used to assess whether epinephrine associates with renalase. Renalase (350 μL of 339 μM in PBS buffer at 25 °C) was injected into a TA Instruments Nano ITC model 5303 microcalorimeter. This sample then had 21 2 μL additions of 1.84 mM epinephrine injected over 2 h. The pattern of heat liberated was then fit to a single independent site model correcting for constant cell volume and injection dilution using NanoAnalyze Data Analysis software (TA Instruments) to obtain a measure of the binding constant for the renalase·epinephrine complex.[31]

Perturbation of the renalase flavin absorption and fluorescence emission spectra in the presence of epinephrine was used as a qualitative measure to evaluate whether the ligand formed a complex with the enzyme that had close association with the FAD isoalloxazine. Absorption spectra of renalase (30 μM) were measured at 25 °C in PBS buffer in the presence and absence of epinephrine (100 μM) using a Cary 3 dual beam spectrophotometer. The free enzyme spectrum was then subtracted from the spectrum obtained in the presence of the ligand to obtain the difference spectrum and accentuate changes. Similarly, fluorescence emission spectra were obtained under the same conditions with excitation at 450 nm. The emission spectrum (475−650 nm) of 4 μM renalase in PBS buffer was recorded in the absence and presence of 40 μM epinephrine using an Hitachi F-4500 spectrofluorometer at 25 °C.

RESULTS

Expression and Purification. Human N-terminally His-tagged renalase isoform 1 was expressed in BL21 DE3 *E. coli* to highest yield at 22 °C. Typically the yield of purified enzyme was 6 mg/L of culture. The enzyme was purified to homogeneity using a cobalt affinity column from which it eluted at approximately 30 mM imidazole and was assessed to be greater than 95% pure by SDS polyacrylamide gel electrophoresis. Consistent with prior reports, the renalase flavin spectrum was red-shifted compared with that of free flavin (Figure 1).[3,22] The two visible maxima of the enzyme bound flavin spectrum occur at 387 and 458 nm with extinction coefficients of $\varepsilon_{387\text{nm}}$ = 10750 M^{-1} cm^{-1} and $\varepsilon_{458\text{nm}}$ = 11330 M^{-1} cm^{-1}. The purified enzyme was stable in PBS buffer, could be stored indefinitely at −80 °C, and did not exhibit diminished activity with thawing. Heterologous expression of renalase in *E. coli* resulted in significant inclusion body accumulation at all temperatures tested. Attempts were made to refold this peptide to form active renalase following the methods of Desir et al.[14] The refolded peptide acquired FAD and was soluble to high concentration but had a cofactor spectrum ostensibly unchanged from free FAD (Figure 1). Moreover, the refolded material did not have the activity described hereafter and was deemed to be misfolded inactive protein.

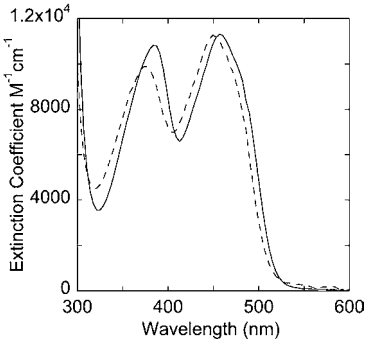

Figure 1. Flavin spectra of native and refolded renalase. Spectra shown are for heterologously expressed renalase from the soluble (solid line) and refolded from the insoluble or inclusion body (dashed line) fractions. The insoluble fraction was refolded using the methods of Desir et al.[14] The extinction coefficients of both forms were determined as described in the Materials and Methods section.

Identification of a Renalase Substrate in NAD(P)H Stock Solutions. A substrate for renalase was detected while validating prior claims that β-NAD(P)H can serve as a renalase

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reductant in the production of adrenochrome from epinephrine.[15] Figure 2 depicts quantitative evidence for a renalase

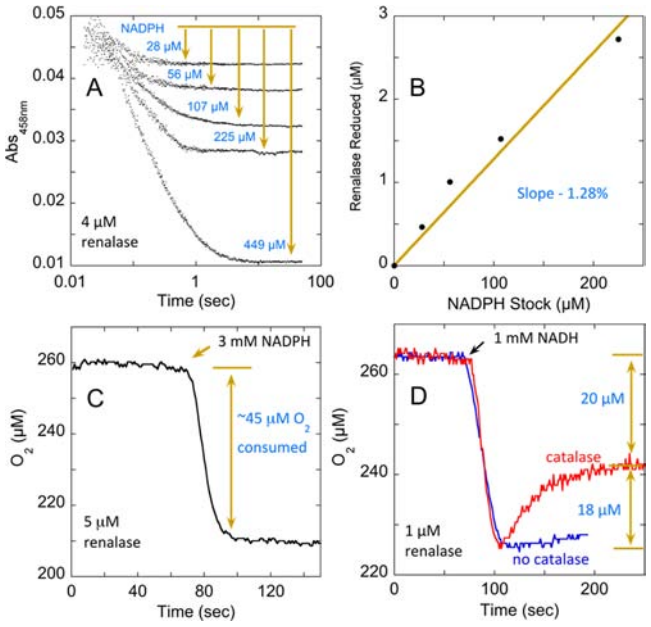

Figure 2. Evaluation of the renalase active fraction in NAD(P)H stock solutions. (A) Stopped-flow traces of fractional reduction of renalase as observed at 458 nm. Renalase (4.0 μM) was mixed under anaerobic conditions with varied concentrations of NADPH as shown. (B) The fraction of NADPH consumed in the reduction in panel A based on the change in extinction coefficient of the renalase flavin cofactor at 458 nm vs the stock concentration of NADPH. (C) Demonstration of multiple turnovers with excess substrate. Five micromolar renalase was added to PBS buffer in a dioxygen electrode and 3 mM of NADPH was added at the arrow. (D) Stoichiometry of the renalase reaction with respect to dioxygen. Renalase (1 μM) was mixed with 1 mM NADH with and without 1 U of catalase. The traces shown in panels C and D are representative traces of typically 3−5 replicates for each observation.

substrate occurring as a 1−4% impurity in β-NAD(P)H solutions. Figure 2A shows that rapid fractional reduction of renalase occurs when the enzyme is mixed with relative high concentrations of NADPH under strict anaerobic conditions. The measured $\Delta\varepsilon_{458nm}$ value (7945 M^{-1} cm^{-1}) for reduction, obtained when the substrate contaminant was added in apparent excess (449 μM NADPH reduction trace), permitted the concentration of the actual substrate at all limiting values to be calculated. When the concentration of fractionally reduced renalase was plotted against the NADPH stock concentrations, a slope of 1.3% was determined (Figure 2B). In a separate experiment, high concentrations of NADPH were added to 5 μM renalase in a dioxygen electrode and the concentration of oxygen consumed was measured (Figure 2C). The amplitude measured in this experiment indicated that the substrate contaminant was present to 1.5% of the total NADPH concentration. Higher apparent substrate fractions (4−5%) were observed with NADH stock solutions (Figure 2D). When catalase was included in these assays close to one-half of the dioxygen consumed by renalase was regained from the catalase disproportionation reaction indicating that hydrogen peroxide is a product of renalase activity. Collectively these data establish multiple characteristics of the renalase reaction. In regard to the contaminant substrate, the proportion in NAD(P)H solutions is small and can vary with the parent molecule suggesting degradation or equilibrium processes result in the substrate accumulation. The complete and monophasic reduction of the flavin cofactor in excess substrate under anaerobic conditions (Figure 2A, 449 μM NADPH trace) suggests that one substrate molecule transfers two electrons to the flavin. The dioxygen electrode assays define that dioxygen is also a substrate for renalase with an observed rate of dioxygen consumption in solutions equilibrated with atmospheric dioxygen (~250 μM) of ~0.5 s^{-1}. The total dioxygen consumption also indicated that multiple (Figure 2C,D) turnovers occurred in the presence of the substrate component of both NADPH and NADH solutions. That the reduction (Figure 2A) and dioxygen uptake (Figure 2C) experiments indicate a similar proportion of substrate in the NADPH stocks suggests that the stoichiometry of the reaction is 1:1 for dioxygen and the NADPH contaminant. Moreover, that we regain 0.5 equiv of O_2 in the presence of renalase and catalase suggests that the product stoichiometry is also 1:1; product/H_2O_2.

HPLC product analysis was used to separate the components present in NADPH solutions and identify the substrate component (Figure 3). HPLC chromatograms were collected

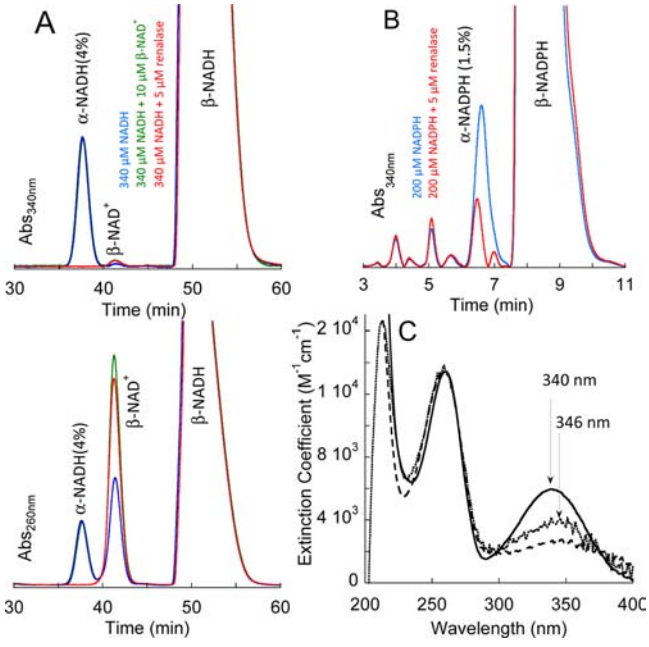

Figure 3. HPLC identification of α-NAD(P)H as the substrate for renalase occurring as a contaminant of β-NAD(P)H solutions. (A,B) Species were separated using a Xterra reverse phase C18 column running isocratically in 10 mM sodium phosphate buffer, pH 7.5. In each, the blue line is the control β-NAD(P)H stock while the chromatograms depicted as a red line were prepared identically to the control sample but were reacted with 5 μM renalase and then filtered to remove the enzyme. The chromatogram depicted with a green line in panel A is the same as the control sample but with 15 μM β-NAD^+ added. (A) Chromatographic separation of NADH solutions detected at 340 nm (upper) and 260 nm (lower-7-fold smaller scale). (B) Chromatographic separation of NADPH solutions detected at 340 nm. (C) Spectra of the renalase substrate species resolved in panels A and B. The spectrum represented with an unbroken line is β-NADPH and was included for reference. The dotted line spectrum was of the substrate peak in panel A. The dashed line spectrum was of the substrate peak in panel B. Spectra were normalized at 260 nm for clarity.

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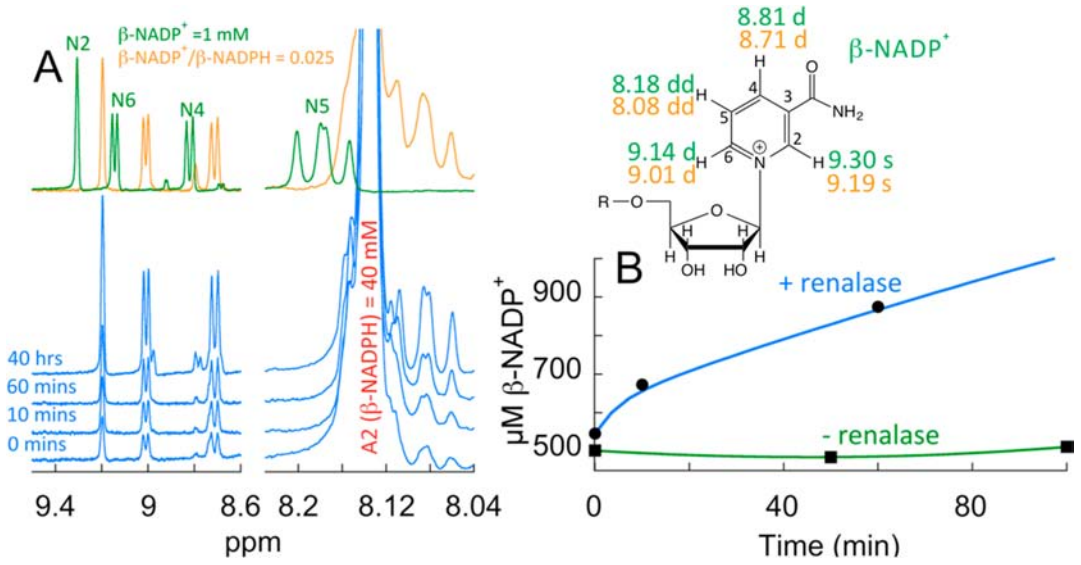

Figure 4. Identification of β-NAD(P)$^+$ as the nicotinamide product of the renalase reaction. (A) ^{1}H NMR spectra, 300 MHz. Blue spectra show the accumulation of resonances when 10 μM renalase was added to a freshly prepared solution of 40 mM β-NADPH in 10 mM sodium phosphate buffered deuterium oxide, pH 7.4. The upper spectra are control samples. For positional assignments, the N designation refers to the nicotinamide base, while A references the adenine base. The green spectrum is that of 1 mM β-NADP$^+$, and the orange spectrum is that of 1 mM β-NADP$^+$ in the presence of 40 mM β-NADPH (both control samples prepared in 10 mM sodium phosphate buffered deuterium oxide, pH 7.4). Assignments of the shifts of the nicotinamide are shown to the right of the control spectra. (B) Relative accumulation of β-NADP$^+$ in the presence and absence of renalase based on the integration of the N2 proton and the known concentration of the β-NADPH in the sample.

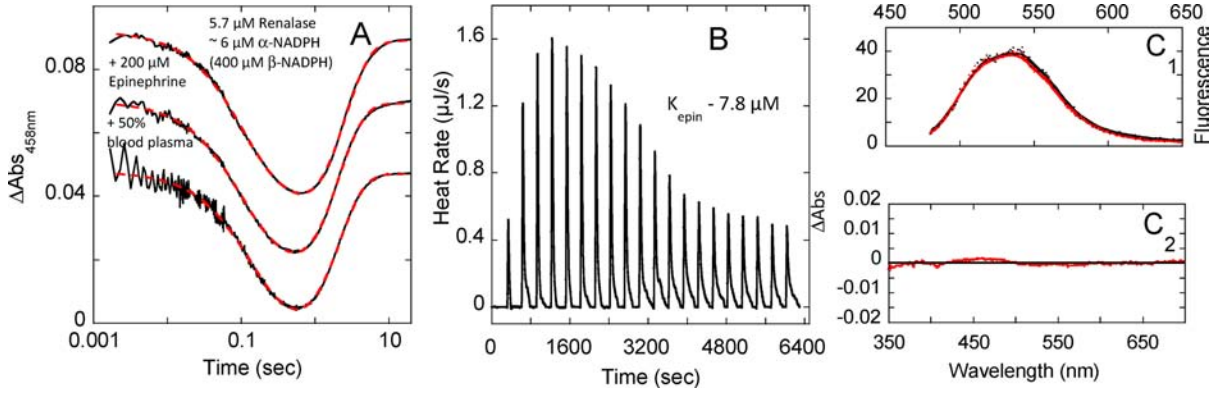

Figure 5. Evaluation of epinephrine as a substrate for renalase. (A) Single turnover of renalase with limiting α-NADPH. Traces were observed at 458 nm when 5.7 μM renalase was reacted with ~6 μM α-NADPH at atmospheric concentrations of molecular oxygen (~250 μM). Dashed red lines indicate fits to eq 1. (B) Isothermal titration calorimetry of 339 μM renalase with 21 2 μL injections of 1.84 mM epinephrine. (C) The extent of spectrophometric perturbation of the flavin spectrum by epinephrine: (C_1) Black line, flouresence emission spectrum of 4 μM renalase when excited at 450 nm; red line, flouresence emission spectrum of 4 μM renalase plus 40 μM epinephrine when excited at 450 nm; (C_2) absorption difference spectra; black line, zero perturbation difference spectrum of 20 μM renalase; red line, difference spectrum (sample − control) for 20 μM renalase plus 200 μM epinephrine.

at known maxima for NADPH and NADH (340, 260 nm). Chromatograms collected at 340 nm indicated respective ~1.5% and 4% contaminants (by area) for NADPH and NADH solutions (Figure 3A,B). This component was largely consumed when the sample was incubated with renalase for 2 min (Figure 3A,B, red chromatograms) while the area of the major components (β-NADPH and β-NADH) remained unchanged. This indicated that the reactive component absorbed light at 340 nm and that this absorption was lost when reacted with renalase. An increase in absorption at 260 nm coinciding with a component already present in the NADH solution was observed in the sample to which renalase was added (Figure 3A, lower). Coinjection of 15 μM of β-NAD$^+$ in the control sample identified the product of the renalase reaction and indicated that the substrate concentration in the unreacted sample was ~13 μM (4.0%). For NADPH and NADH samples, the spectra of the substrate components (Figure 3C) exhibited maxima at 260 and 346 nm suggesting that the nicotinamide and adenine bases are intact in the substrate molecule. The loss of the 346 nm absorption of the substrate component when reacted with renalase is consistent with the dihydropyridyl base transferring a hydride equivalent to enzyme cofactor.

Red-shifted nicotinamide spectra of the active components of NADPH and NADH solutions is characteristic of α-NAD(P)H molecules.[30,32] Among the numerous decay paths possible for pyridine nucleotides are anomerization equilibria that bring about the accumulation of a small fraction of the α-

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dihydronicotinamides in dissolved β-NAD(P)H solutions[33] (Scheme 1). Anomerization is only possible for the reduced form because the ribose ring-open Schiff base intermediate can form only when electrons are delocalized from the electron-replete dihydropyridyl ring; the oxidized forms are thus configurationally stable. Reduced nicotinamide nucleotides are one of the two naturally occurring nucleotides that exhibit anomerization in solution, the other being pseudouridine nucleotide.[34] The α/β equilibrium constants for NADPH and NADH are reported as 0.015 and 0.11 respectively,[30] largely consistent with the ∼1.5% and ∼5% fractions observed for the experiments described above.

NMR was used to confirm the product of the renalase reaction. The oxidized nicotinamide proton resonances of the α and β forms of NAD(P) are readily discerned by NMR.[19] With respect to β-NAD(P), the resonances of protons of the α-nicotinamide moiety move to higher field while the discriminating proton attached to the anomeric carbon of the nicotinamide ribose moves downfield. Figure 4A depicts proton NMR spectra for the accumulation of β-$NADP^+$ from a stock solution (40 mM) of β-NADPH in the presence of renalase (blue spectra). In such a reaction, α-NADPH is expected to be consumed by renalase as a burst of product formation before the reaction becomes limited by both available dioxygen and the rate of anomerization. What is observed is an ∼200 μM burst in product accumulation that did not occur in the absence of renalase (Figure 4B). That the observed product nicotinamide resonances are shifted upfield relative to those of β-$NADP^+$ alone (Figure 4A, green and blue spectra) indicates only the influence of the high concentration of β-NADPH in these samples. When the proton resonances and coupling constants are compared with those of a control sample having the same ratio of β-$NADP^+$ to β-NADPH, the product and control spectra coincide (Figure 4A, orange and blue spectra), indicating that the product of the reaction is β-$NADP^+$.

Evaluation of Epinephrine as a Substrate for Renalase. The substrate for renalase has been the subject of some dispute since the discovery of the enzyme in 2005.[16,20,35] Figure 5 summarizes the data obtained for three approaches used to assess the response of renalase to the presence of epinephrine. In Figure 5A, non-pseudo-first-order single-turnover reactions of renalase with limiting α-NADPH and atmospheric dioxygen (observed at 458 nm) indicated reduction and reoxidation of the flavin cofactor. Under the conditions used, these data could be fit to three exponential phases without significant systematic error in the residuals (11.6 s^{-1}, 1.63 s^{-1}, and 0.54 s^{-1}). However, the number of phases, the values for rate constants, and absorbance amplitudes are only apparent measures of the single turnover kinetics that provide a means to compare the data in the presence of and absence of epinephrine. It has been suggested that renalase obtains reducing equivalents from β-NADPH and then catalyzes a net two-electron oxidation of epinephrine to form adrenochrome, a process that would mobilize four electrons.[15] The addition of 200 μM epinephrine to single turnover reactions with α-NADPH and dioxygen did not change the observed kinetics (11.0 s^{-1}, 1.82 s^{-1}, and 0.66 s^{-1}) indicating that no rate constant is significantly altered and no new intermediates accumulate in response to the catechol. It has been suggested that renalase is only activated in blood and is isolated as a relatively inactive pro-renalase.[9] In order to assess whether some additional soluble component of blood is required to activate renalase to consume epinephrine, the experiment was repeated in 50% fresh cell-free human blood plasma. This condition also did not significantly alter the observed single turnover kinetics (12.1 s^{-1}, 1.90 s^{-1}, 0.64 s^{-1}). The conclusion is that epinephrine is not catalytically consumed by renalase as isolated and that no soluble ligand in blood activates the enzyme to display this activity.

To evaluate whether renalase binds epinephrine, the enzyme was titrated with epinephrine in an isothermal titration calorimeter (Figure 5B). The data obtained suggest an exothermic binding event with a dissociation constant for the renalase·epinephrine complex of ∼8 μM. This complexation is either adventitious (and coincidental) or is a binding event whose link to physiology is yet to be established. The lack of influence of epinephrine on the observed kinetics of reduction and reoxidation of renalase by α-NADPH implies that the binding of epinephrine does not occur in the active site of the enzyme. To test this hypothesis, spectrophotometric observations using the flavin absorption and emission spectra as sensitive measures of ligand proximity were undertaken (Figure 5C). These experiments show no appreciable perturbation of the flavin environment in the presence of epinephrine concentrations at least 4-fold higher than the measured binding constant, suggesting that the binding site for epinephrine is distant from the active site.

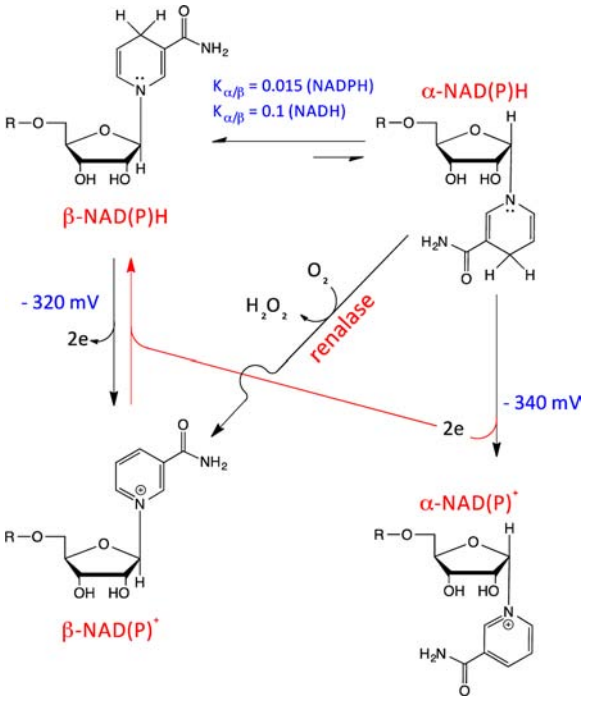
The accumulation of adrenochrome from epinephrine can be observed at 480 nm.[18] Our experiments indicated that the initial rate of oxidation of epinephrine to form adrenochrome in atmospheric oxygen at 25 °C in PBS buffer was ostensibly zero for all concentrations tested. The addition of freshly dissolved β-NADPH did not bring about adrenochrome formation nor did the addition of 10 μM renalase (data not shown). As such it was concluded that renalase does not utilize β-NAD(P)H (or α-NAD(P)H) to catalyze a reaction that forms adrenochrome.

■ DISCUSSION

Eight years after its initial discovery, we have identified an activity for human renalase as an α-NAD(P)H oxidase/anomerase. This activity is the first demonstration of a potential physiological role for α-NAD(P)H in mammals and the first example of a flavoprotein catalyzing an anomerization reaction. This discovery runs contrary to the persistent assertion that renalase catabolizes adrenergic catecholamines. Renalase has often been described as "MAO C", the third monoamine oxidase[14,15,36] that oxidizes the catechol ring rather than the distal amine. We suggest that epinephrine and other catechols have most often been reported as the substrate for renalase because they exhibit instability at or above neutral pH values and form chromophoric products (aminochromes) that may be easily taken for slow catalytic activity.[15] Our data suggest that epinephrine has no influence on the activity of the enzyme.

It may be possible to reconcile our observations with the majority of earlier reports of adrenochrome formation by renalase. *In vitro* catalytic adrenochrome production by renalase is solely for the refolded renalase peptide.[14] In our hands, this form of the protein exhibits none of the α-NADPH oxidase/anomerase activity described herein. It is thus tentatively proposed that the refolded form may have a solvent exposed flavin isoalloxazine ring (Figure 1) that due to the difference in reduction potentials (−320 vs −207 mV) will be reduced by NADPH in a slow steady-state reaction that forms H_2O_2 autocatalytically. This molecule will readily oxidize epinephrine to initially form a quinone species prior to nucleophilic attack

Scheme 2

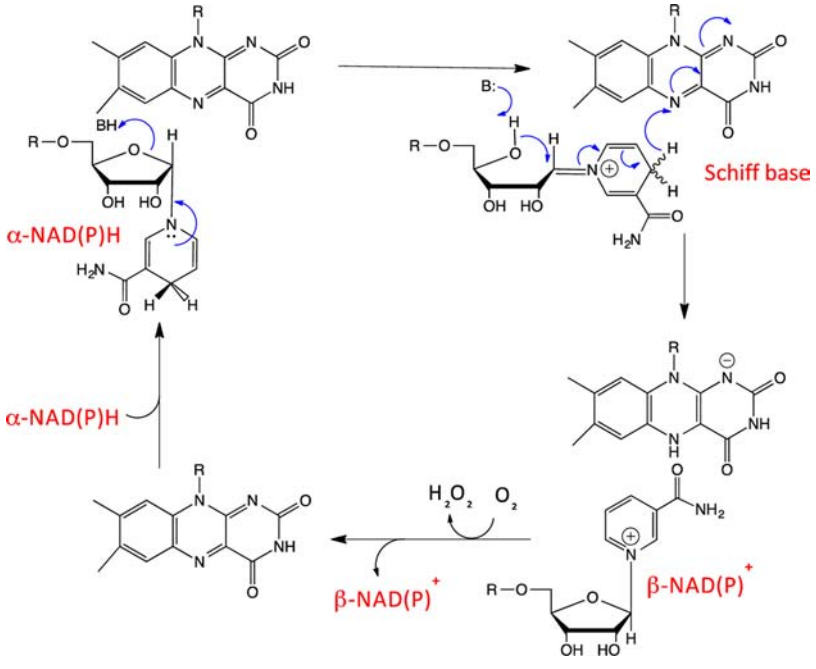


by the distal amine of the side chain to yield the adrenochrome product. The natively folded renalase enzyme does not promote this chemistry to any significant extent because it selects against binding of the β-form from the NAD(P)H reductant pool (Figures 1−3). Moreover, it may be possible to observe the accumulation of aminochromes with natively folded renalase in the presence of reduced nicotinamide dinucleotides when long incubations are employed because nonenzymatic anomerization will form α-NAD(P)H as a minor component resulting in the formation of product H_2O_2 and hence promote the ensuing nonenzymatic oxidation and cyclization of the catechol.

From an experimental perspective α-NAD(P)H molecules are highly elusive. The apparent rate constants to attain equilibrium from the α-NAD(P)H anomer are on the order of 10^{-5}−10^{-6} s^{-1}.[30] As such the concentration of pure α-NADPH is altered within hours of a solution being prepared, to some extent undermining the systematic use of the pure substrate in activity assays. The use of the β-anomer to obtain the α-form requires prolonged incubation to reach anomer equilibrium and verification of the α-fraction before use as a stock solution but has the advantage of supplying a constant α/β ratio throughout the period of observation. While α-NAD(P)H will invariably be the minor component, it must be assumed that renalase is highly discriminatory for the α-anomer given that it was destined to function in an environment in which the β-anomer is predominant.

More evidence is needed to define the means by which the activity of renalase transmits a signal to the circulatory system to induce the reported vasodilation. However, this oxidase/anomerase activity may have a core physiological role that is separate and more pervasive than blood pressure modulation. The necessity for renalase to harbor two functionalities may arise from the fact that α-NAD(P)$^+$ molecules are metabolically isolated. In Scheme 2, the α/β equilibria are depicted with the redox pathways for both anomers of NAD(P)H molecules. The inherently lower reduction potential of α-NAD(P) molecules and the lack of participation in other metabolic pathways dictates that there would be an inexorable loss of nicotinamide dinucleotide cofactors as α-NAD(P)$^+$. Without considering other redox partners, the anomer equilibrium and redox potential difference would predict that ∼50% of the β-NAD(P)H would ultimately accumulate as α-NAD(P)$^+$.[19] If renalase were not to catalyze oxidation in addition to anomerization, these molecules would accumulate and deplete both the total concentration of NAD(P)H molecules and the normal NAD(P)H/NAD(P)$^+$ ratio. The α-NAD(P)H oxida-

Scheme 3



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tion couples the reaction to dioxygen reduction providing a large driving force that in concert with the anomerization activity recycles α-NAD(P)H molecules back to the β-pool maintaining the α-NAD(P)H concentration near zero.

This activity for renalase raises numerous chemical and physiological questions. From an enzyme chemistry perspective, hydride transfer to a flavin is not unusual, though the timing of this process with respect to the anomerization reaction is highly intriguing. While it may be reasonable to conclude that anomerization occurs after reduction of the FAD cofactor in order that the enzyme be selective for α-NAD(P)H, this raises the question as to what redox state of renalase catalyzes the anomerization. That is, is α-NAD(P)$^+$ (or β-NAD(P)$^+$) a substrate for the reduced or oxidized enzyme and if the reduced enzyme catalyzes anomerization of the oxidized nicotinamide dinucleotide how is the ribose ring cleaved if the otherwise requisite schiff base/ring open intermediate can no longer form? A hypothetical mechanism that circumvents these apparent conundrums is depicted in Scheme 3. In this mechanism, the oxidized enzyme is selective for the α-NAD(P)H substrate and first promotes the formation of a ring open ribose/Schiff base intermediate. It is then this intermediate that reduces the flavin, concomitantly forming the β-NAD(P)$^+$ product prior to reoxidation.

This research does not address the manner in which this newly identified activity of renalase is imparted *in vivo* in regard to vasodilation. It may be ventured that the observed decrease in blood pressure and slowing of the heart rate is not a result of diminishment of either of the substrates or the accumulation of the β-NAD(P)$^+$ product as each of these molecules are ubiquitous in mammalian circulation. It therefore seems reasonable to implicate H_2O_2 as the signal for vasodilation arising from renalase activity.[37−39]

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Notes

The authors declare no competing financial interest.

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