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Engineering of *Giardia lamblia* NADH Oxidase: A Single Point Mutation Yields an Efficient Dual-Specific Biocatalyst for Cofactor Regeneration

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SECTION

Microbial and Enzyme Technology

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ABSTRACT

Water-forming NADH oxidases (NOXs) are valuable biocatalysts for *in situ* cofactor regeneration; however, most wild-type NOXs exhibit strict specificity for either NADH or NADPH, limiting their general applicability in redox biotransformations. Although complete reversal of cofactor specificity has been engineered in several NOXs, generating a balanced, "dual-specific" catalyst capable of efficiently utilizing both cofactors is highly desirable for industrial applications. Here, we report the rational engineering of the water-forming NOX from *Giardia lamblia* (GINOX). Combining structural modeling with evolutionary sequence alignments, we identified three key residues within the cofactor specificity loop predicted to dictate coenzyme discrimination. Site-directed variants (D180A, G181R, and S182H) were expressed, purified, and structurally characterized by circular dichroism and intrinsic fluorescence spectroscopy, confirming that global protein folding was preserved. Steady-state kinetic analysis revealed that S182H exerted minor effects on catalysis, whereas G181R completely inverted selectivity toward NADPH, albeit at the expense of catalytic rate. In contrast, the single D180A substitution significantly enhanced NADPH utilization while maintaining high catalytic efficiency toward NADH. Electrostatic surface potential mapping confirmed an enlarged, less negatively charged active-site pocket in the D180A variant. Overall, this study demonstrates that a single point mutation guided by *in silico* modeling can yield a versatile, dual-specific biocatalyst ideal for flexible industrial cofactor regeneration.

INTRODUCTION

Oxidoreductases are essential biocatalysts for the sustainable synthesis of active pharmaceutical ingredients and complex chiral molecules. However, the economic viability of these enzymatic transformations is frequently limited by their reliance on stoichiometric amounts of expensive nicotinamide cofactors, namely NAD⁺ and NADP⁺ [Shah 2025, Zhou 2025]. To overcome this thermodynamic bottleneck, the development of robust *in situ* cofactor regeneration systems is critical. Among the available enzymatic methods, water-forming NADH oxidases (NOXs) have emerged as highly efficient tools. These FAD-dependent enzymes utilize molecular oxygen as an electron acceptor to recycle the oxidized cofactor, producing water as the sole byproduct [Shah 2025]. This mechanism effectively circumvents the toxicity and premature enzyme deactivation typically associated with hydrogen peroxide-producing oxidases.

Despite their catalytic advantages, wild-type NOXs typically exhibit a strict structural preference for either NADH or NADPH. For instance, the enzymes from bacteria like *Streptococcus pyogenes* [Gao 2012], *Lactobacillus brevis* [Titov 2016], and *Lactobacillus rhamnosus* [Li 2019], possess a strong natural preference for NADH, whereas NOXs from *Lactobacillus sanfranciscensis* [Lountos 2006], *Lactobacillus reuteri* [Gao 2019] and *Thermococcus kodakaraensis* [Nisar 2013], efficiently utilize NADPH. Because industrial reductases and anabolic pathways primarily require NADP⁺, engineering the cofactor specificity of NADH-preferring NOXs has become a primary objective in protein design [[Li 2019, Cracan 2017]. Previous engineering efforts have largely focused on the complete reversal of coenzyme selectivity—aiming to switch an enzyme's preference entirely from NADH to NADPH. However, from a biotechnological perspective, a "dual-specific" enzyme capable of high catalytic efficiency with both cofactors is highly desirable, as it would simplify reaction systems and reduce costs across diverse synthetic pathways [Petschacher 2014].

In this regard, the water-forming NOX from *Giardia lamblia* (GINOX) can use both cofactors, but with a high natural preference for NADH. Since it has been shown that recombinant GINOX can be produced with high yields and purity [Castillo-Villanueva 2016] and its kinetic stability can be greatly improved by medium additives, this enzyme represents a promising candidate for biotechnological applications [Castillo-Villanueva 2021]. However, the absence of an experimentally determined crystal structure has previously hindered rational engineering efforts to expand its substrate scope.

In this study, we combined high-confidence *in silico* 3D modeling via AlphaFold with multiple sequence analysis to identify the molecular determinants of cofactor specificity in GINOX. The analysis suggests that electrostatic repulsion within the cofactor specificity loop acts as the primary barrier to NADPH utilization. By targeting this region with site-directed mutagenesis, we aimed to relieve this electrostatic hindrance without compromising the native catalytic architecture. Here, we report the structural and steady-state kinetic characterization of engineered GINOX variants, demonstrating that a single, targeted amino acid substitution is sufficient to generate a highly efficient, dual-specific biocatalyst.

MATERIALS AND METHODS

General Reagents and Materials

Analytical-grade buffers, salts, and substrates were purchased from MilliporeSigma. Molecular biology reagents were obtained from Promega and Thermo Fisher Scientific. Oligonucleotide synthesis and DNA sequencing services were provided by the *Unidad de Síntesis y Secuenciación de ADN* at the *Instituto de Biotecnología*, UNAM.

Bioinformatics Analysis

NADH oxidase sequences were retrieved from the National Center for Biotechnology Information (NCBI) Protein database. Multiple-sequence alignment was

performed using ClustalX 2.0 [Larkin 2007] and visualized with BioEdit [Hall 1999]. The GINOX 3D model was generated using the AlphaFold server [Varadi 2024]. Visualization, structural alignment, distance measurements, and electrostatic surface visualization were performed in Discovery Studio Visualizer (Accelrys Software Inc., San Diego, CA, USA).

Site-Directed Mutagenesis and Protein Purification

The amplification, cloning, expression, and characterization of wild-type (WT) GINOX were previously reported [Castillo-Villanueva 2016]. In accordance with those protocols, the GINOX gene cloned into the pET23b plasmid was used as the template for this study. The D180A, G181R, and S182H mutations were introduced via site-directed mutagenesis. The mutagenic oligonucleotides used were: D180A: Fw 5'-CTTATTCCCGGCTCTGGTAGGAT-3' and Rv 5'-ATCCTACCAGAGCCGGCCAATAAG-3'; G181R: Fw 5'-GCTTATTGACGCCTCTGGTAGGAT-3' and Rv 5'-ATCCTACCAGAGGCGTCAATAAGC-3'; and S182H: Fw 5'-GCTTATTGACGGCCCATGGTAGGAT-3' and Rv 5'-ATCCTACCATGGCCGTCATAAGC-3'. The mutant proteins were expressed in *Escherichia coli* BL21(DE3) and purified by immobilized metal affinity chromatography (IMAC), as previously described [Castillo-Villanueva 2016]. Protein concentration was determined by the Bradford assay using bovine serum albumin (BSA) as a standard.

Spectroscopic Analysis

Circular dichroism (CD) and intrinsic fluorescence spectroscopy were performed essentially as described [Castillo-Villanueva 2016]. Briefly, far-UV CD spectra (190–280 nm) were recorded for 0.1 mg/mL protein samples in 50 mM phosphate buffer (pH 7.4) using a Jasco J-810 spectropolarimeter. Fluorescence spectra (excitation at 280 nm; emission from 300 to 450 nm) were obtained using a Perkin-Elmer LS-55 spectrometer under identical conditions. All measurements were performed in triplicate, and buffer blanks were subtracted from the experimental samples.

Kinetic Characterization

Steady-state kinetic measurements of NADH oxidase activity were performed spectrophotometrically as previously described [Castillo-Villanueva 2016], with the modification that medical-grade oxygen was used to saturate the reaction media [Castillo-Villanueva 2021]. All experiments were performed in triplicate. Experimental data points represent the mean $\pm$ standard deviation of three independent measurements. Data were fitted to the Michaelis-Menten model using non-linear regression analysis. The concentrations of NADH and NADPH were varied for each mutant to accurately determine their respective kinetic parameters.

RESULTS

Engineering Strategy and *In Silico* Modeling

Since the crystal structure of GINOX is currently unavailable, a 3D model was generated using AlphaFold [Varadi 2024]. The model exhibited high reliability, with a per-residue confidence score (pLDDT) exceeding 90 for the majority of the structure. To validate this model, it was superposed onto the crystal structure of the orthologous water-forming NAD(P)H oxidase from *L. sanfranciscensis* (PDB: 2CDU), which contains bound ADP [Lountos 2006]. The structural alignment yielded a root-mean-square deviation (RMSD) of 1.43 Å, with primary differences localized to external loops distant from the active site (Fig. 1A). Accordingly, the GINOX model was deemed suitable for further analysis.

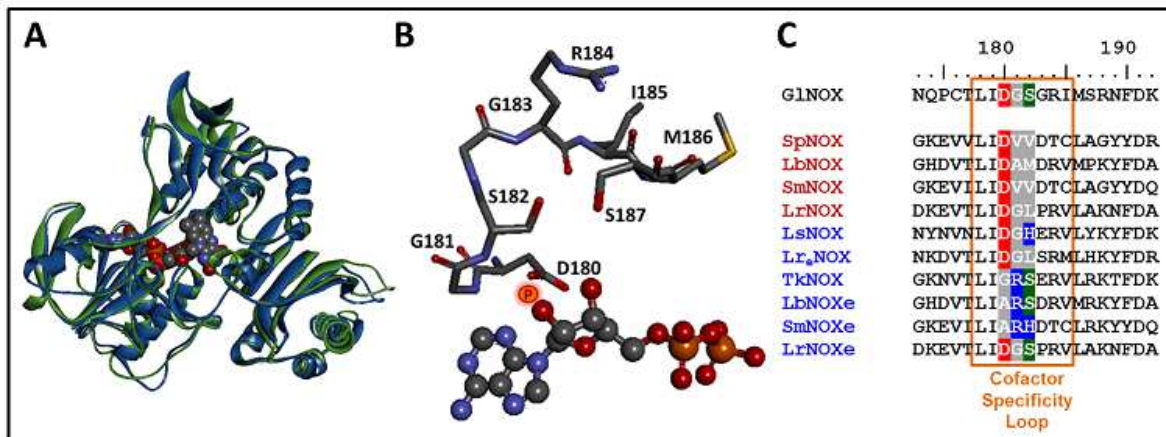


Fig. 1 Structural analysis of the NADH oxidase model and multiple sequence alignment. A) Structural superposition of GINOX and LsNOX. shown as blue and green ribbons, respectively. The ADP molecule bound at the active site is depicted as a space-filling model using the standard CPK coloring scheme. B) Structural model of the cofactor specificity loop, amino acid residues are shown as stick models and ADP as a ball and stick model. C) Sequence alignment around the cofactor specificity loop (orange box). NADH preferring enzymes are shown in red whereas NADPH using enzymes are in blue.

To identify residues responsible for coenzyme specificity, a multiple sequence alignment was performed using NOX enzymes with varying selectivity. Enzymes from *S. pyogenes* (SpNOX) [Gao 2012], *L. brevis* (LbNOX) [Titov 2016], *S. mutans* (SmNOX) [Higuchi 1993], and *L. rhamnosus* (LrNOX) [Li 2019] possess a natural strong preference for NADH. In contrast, enzymes from *L. sanfranciscensis* (LsNOX) [Lountos 2006], *L. reuteri* (Lr_eNOX) [Gao 2019] and *T. kodakaraensis* (TkNOX) [Nisar 2013], efficiently utilize NADPH. We also included previously engineered variants of LbNOX, SmNOX, and LrNOX (here named LbNOXe, SmNOXe, and LrNOXe, respectively), where switching of selectivity toward NADPH has been engineered [Li 2019, Cracan 2017, Petschacher 2014].

Our analysis focused on the "cofactor specificity loop" (D180 to S187 in GINOX). The analysis of the structural model indicated that residues 180-182 are within 4Å° of the

C2' and C3' hydroxyls of the ribose ring and the putative phosphate group position of NADPH (Fig. 1B). From the alignment (Fig. 1C), there doesn't seem to be a clearly specific sequence signature for coenzyme specificity, however, some patterns could be extracted.

D180 in GINOX and other NADH using enzymes is substituted preferentially by small neutral residues in NADPH-using enzymes, suggesting that the negatively charged aspartic acid causes electrostatic repulsion with the NADPH phosphate group. Furthermore, positions 181 and 182 are predominantly apolar residues in NADH-preferring enzymes, which are typically replaced by positively charged or polar residues in enzymes that utilize NADPH. Based on these observations, we designed the D180A, G181R, and S182H mutations to modulate cofactor recognition.

Production and Structural Characterization of GINOX Mutants

The mutants were successfully generated via site-directed mutagenesis and verified by full gene sequencing. Expression and purification of the 3 proteins was similar to GINOX-WT [Castillo-Villanueva 2016]; with yields of approximately 20 mg/L and purity exceeding 90%, as indicated by SDS-PAGE (Fig. 2A). Far-UV circular dichroism (CD) spectra showed that all mutants maintained a secondary structure nearly identical to the WT, although a minor signal reduction was noted for G181R (Fig. 2B). Intrinsic fluorescence data further supported these findings, showing only minimal alterations in the microenvironment of aromatic residues (Fig. 2C). The most notable change occurred in the G181R mutant, which exhibited a 4-nm blue-shift in its emission maximum and a 5% decrease in intensity. Overall, these results confirm that the introduced mutations did not induce significant structural disruptions, allowing for a reliable comparison of their kinetic properties.

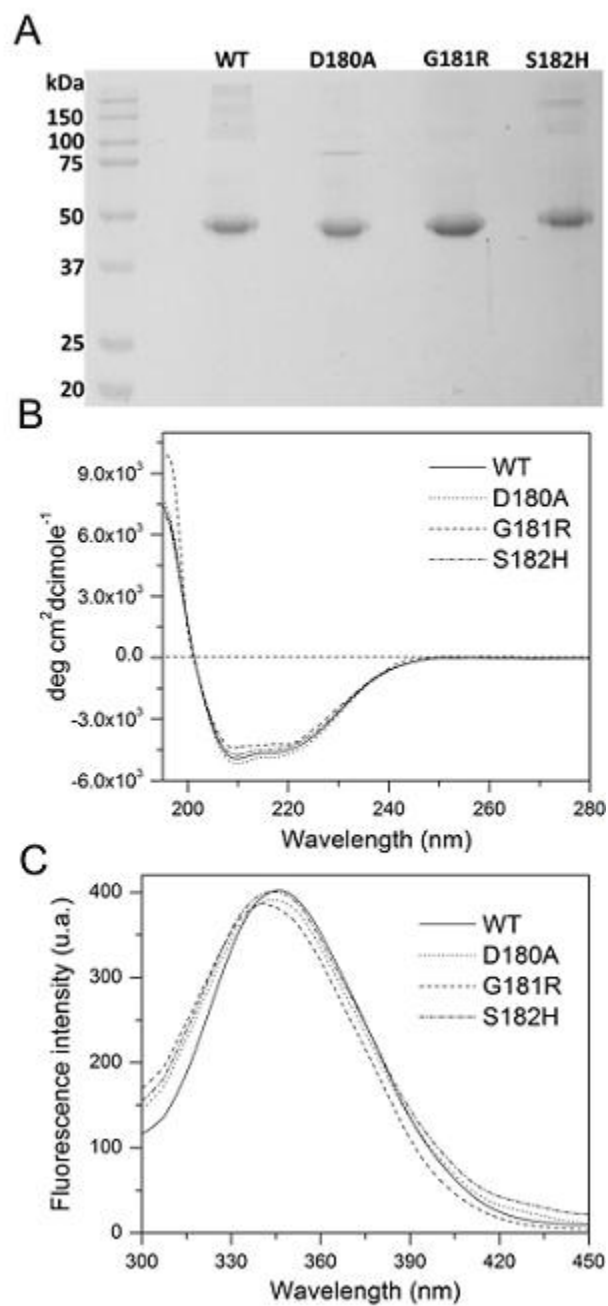


Fig. 2 Production and Structural Characterization of WT GINOX and Mutants. A) Proteins were purified by IMAC chromatography and analyzed in 12% SDS-PAGE. B) Far-UV circular dichroism and C) Intrinsic fluorescence spectra of GINOX WT and mutants.

Kinetic Characterization

Mutants followed Michaelis-Menten kinetics for both substrates as the parental enzyme (Fig. 3), but kinetic constants varied differentially (Table 1). All mutations reduced V_{max} , but the reduction was higher for G181R. In contrast, changes in K_m were more complex. Regarding the affinity for NADH the changes were minor; in the D180A and G181R mutants the K_m was reduced by a third, whereas for S182H increases by the same extent. For NADPH, the K_m decreased 6-fold in the D180A mutant and 21-fold in G181R, and increases 1.3-fold in S182H (Table 1).

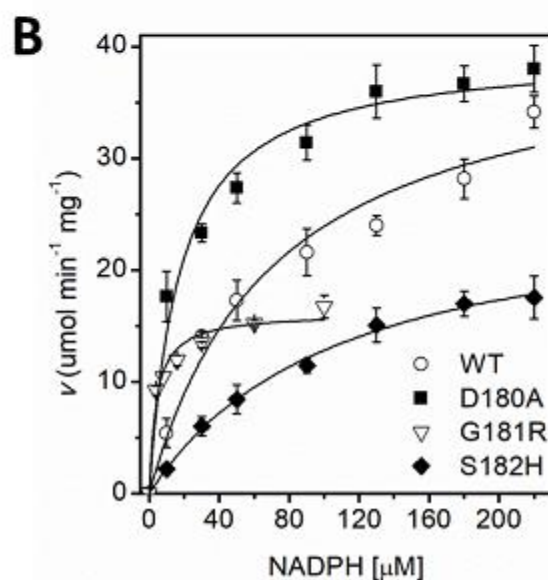
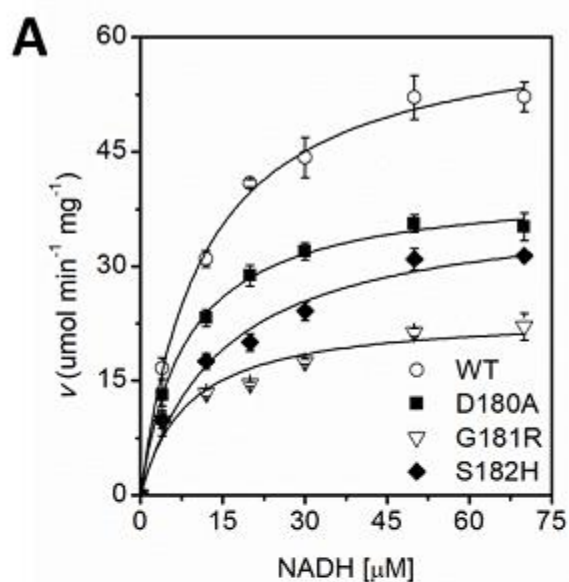


Fig. 3 Functional characterization of NADH oxidase WT and mutants. Steady-state kinetics with A) NADH and B) NADPH as substrates. Initial velocity data were measured at the substrate concentrations indicated in the abscissa axis. Each point represents the mean of three independent replicates $\pm$ standard deviation (bars). The line represents the fit of the data to the Michaelis–Menten equation by non-linear regression calculations.

Table 1. Kinetic constants for NADH oxidase WT and mutants. The data are derived from the analysis shown in Figure 1.

	NADH		NADPH	
	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	K_m (μM)	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	K_m (μM)
WT	62.2 ± 5.3	11.3 ± 2.0	43.4 ± 2.0	83.0 ± 6.1
D180A	40.1 ± 1.4	8.2 ± 1.2	38.1 ± 2.4	13.1 ± 3.3
G181R	23.7 ± 0.5	8.5 ± 0.7	16.1 ± 0.8	4.0 ± 0.5
S182H	38.1 ± 1.4	14.9 ± 0.7	26.3 ± 0.9	111.2 ± 9.9

While it is true that the analysis of individual kinetic constants can be useful for the evaluation of the functional effects of enzyme mutations, a kinetic constant that summarize the negative and positive effects on substrate binding and catalysis will be easy to analyze; therefore, we calculated the second-order rate constant k_{cat}/K_m (Table 2). The k_{cat}/K_m ratio describes the substrate affinity and catalytic rate in a single constant; a higher k_{cat}/K_m is indicative of a better enzymatic performance [Koshland 2002]. To avoid misconception of this term and following the reasoning of Koshland [2002], k_{cat}/K_m is here referred as the performance constant. The relation between the performance constants for NADH and NADPH ($[k_{cat}/K_m]_{\text{NADH}} / [k_{cat}/K_m]_{\text{NADPH}}$) was defined as the discrimination factor (Table 2); values greater than 1 indicate the enzyme's preference for NADH, while values less than 1 indicate selectivity for NADPH; a value of 1 shows that the enzyme uses both substrates equally.

Table 2. Performance constants (k_{cat}/K_m) and discrimination factors for NADH oxidase WT and mutants

	NADH	NADPH	
	k_{cat}/K_m ($M^{-1} s^{-1}$)	k_{cat}/K_m ($M^{-1} s^{-1}$)	Discrimination factor*
WT	4.4×10^6	3.3×10^5	13.3
D180A	3.9×10^6	2.4×10^6	1.6
G181R	2.2×10^6	3.3×10^6	0.7
S182H	2.0×10^6	1.9×10^5	10.5

* Specificity constant of NADH vs NADPH (k_{cat}/K_m NADH / k_{cat}/K_m NADPH)

Unlike previous engineering efforts in other NOX enzymes that aimed to completely reverse selectivity; our goal was to design a variant with high efficiency for both cofactors. The WT enzyme exhibited a high NADH preference with a discrimination factor of 13.3. The G181R mutation effectively reversed this selectivity (factor: 0.7) but at the cost of a 50% reduction in NADH activity. The S182H mutation showed a slight decrease in discrimination (10.5) but resulted in a twofold drop in performance for both substrates. The most significant finding was observed with the D180A mutant, which achieved a balanced performance. With a discrimination factor of 1.6, this variant significantly boosted NADPH efficiency while maintaining high NADH activity.

These results align with the structural hypothesis that neutralizing the negative charge at position 180 reduces electrostatic repulsion with the phosphate group of NADPH. Analysis of the surface electrostatic potential map in the active site vicinity revealed an increase in cavity volume and a reduction in negative charge density in the D180A mutant compared to the WT (**Fig. 4**).

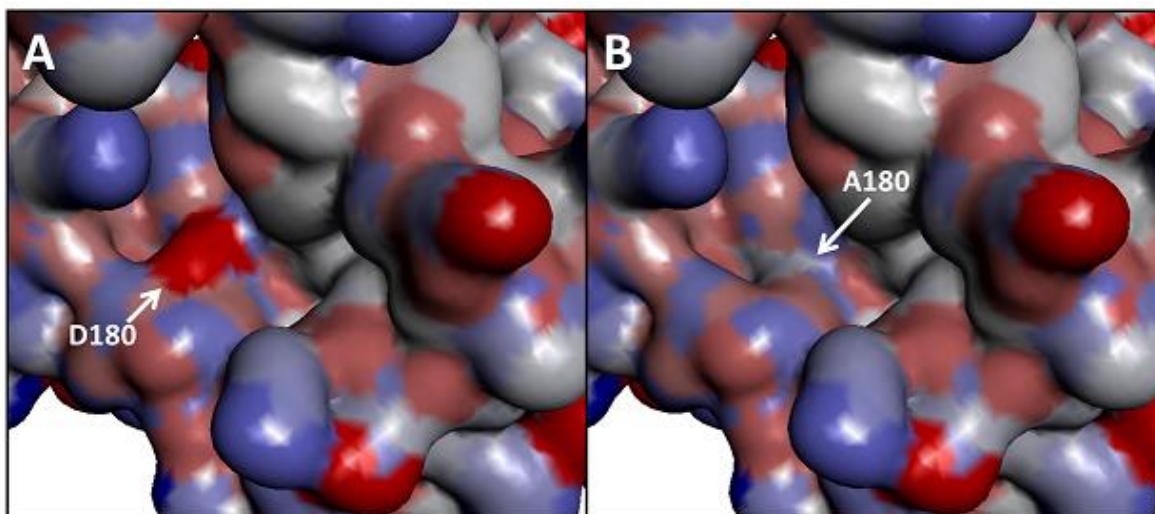


Fig. 4 Surface electrostatic potential map of GINOX. The region in the vicinity of the catalytic site in the WT (A) and D180A mutant (B) is shown.

While engineered mutants from *L. brevis* [Cracan 2017] and *S. mutans* [Petschacher 2014] required multiple mutations to achieve selectivity shifts, the single D180A substitution in GINOX successfully expanded the enzyme's versatility, creating a dual-specific catalyst capable of operating efficiently with both NADH and NADPH. Selectivity shifting from NADH to NADPH was achieved by a single mutation (L179S) in LrNOX [Li 2019] but this change is not analog to the D180A, indicating that there does not appear to be a sequence signature for selectivity in NOX enzymes.

DISCUSSION

The identification of the molecular determinants for cofactor specificity in GINOX was achieved through a synergistic approach combining multiple sequence alignment and 3D structural modeling. This approach revealed that D180 is positioned within 4 Å of the putative 2'-phosphate group of NADPH, supporting the hypothesis that electrostatic repulsion is a main barrier to NADPH utilization in the wild-type enzyme, and suggest that an amino acid change for a small neutral residue could lower this barrier. This dual-strategy approach proved to be effective, demonstrating that in the absence of

experimental crystal structures, the integration of evolutionary sequence data with high-confidence *in silico* models can successfully guide the engineering of new catalytic properties.

Previous research on NOX engineering has focused on the complete reversal of coenzyme selectivity, often aiming to switch an enzyme's preference entirely from NADH to NADPH [Li 2019. Cracan 2017]. However, from a biotechnological perspective, a "dual-specific" enzyme—capable of high efficiency with both cofactors—is often more desirable. In contrast with the work on SmNOX, where extensive mutagenesis experiments were necessary for achieve an enzyme with high activity for both cofactors [Petschacher 2014], our results demonstrate that the single D180A mutation in GINOX achieves this balance remarkably well. This suggests that the active site architecture of GINOX possesses an inherent structural plasticity that can be exploited with minimal genetic intervention.

CONCLUSION

The development of an efficient, dual-specific NADH oxidase has implications for biocatalysis and metabolic engineering. In industrial processes involving the regeneration of oxidized cofactors (NAD^+ and NADP^+); the use of a single, versatile enzyme may simplify reaction systems and reduce costs in synthetic pathways, regardless of the specific cofactor requirement. In addition to its substrate plasticity, GINOX produces only water as byproduct, thus being considered an innocuous regeneration system. Finally, as the kinetic stability of GINOX can be improved by soluble additives, GINOX stands out for its biotechnological potential.

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AUTHOR CONTRIBUTIONS

Adriana Castillo-Villanueva and Jesús Oria-Hernández contributed to the study conception and design. Data collection was performed by Adriana Castillo-Villanueva and Ricardo Pérez-Velázquez. Formal analysis and presentation were performed by Adriana Castillo-Villanueva, Ricardo Pérez-Velázquez and Jesús Oria Hernández. The first draft of the manuscript was written by Jesús Oria Hernández and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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DECLARATIONS

Competing interests

The authors have no relevant financial or non-financial interests to disclose.