



Article

A Unique Insertion Loop Facilitates Tight NAD⁺ Binding in Nicotinoprotein: Insights from In Vitro Loop Engineering and In Silico Studies

Houcheng Xue ¹, Takumi Yanase ¹, Junko Okuda-Shimazaki ¹, Haruka Kawai ¹, Daimei Miura ^{1,2}, Ryutaro Asano ¹, Kazunori Ikebukuro ¹, Koji Sode ³ and Wakako Tsugawa ^{1,*}

¹ Department of Biotechnology and Life Science, Graduate School of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan; xhcbb.xhcbb@gmail.com (H.X.); yanase.01210@gmail.com (T.Y.); junkoshimazaki@go.tuat.ac.jp (J.O.-S.); s246007v@st.go.tuat.ac.jp (H.K.); daimei@go.tuat.ac.jp (D.M.); ryutaroa@cc.tuat.ac.jp (R.A.); ikebu@cc.tuat.ac.jp (K.I.)

² Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, 3-8-1 Harumi-cho, Fuchu, Tokyo 183-8538, Japan

³ Lampe Joint Department of Biomedical Engineering, The University of North Carolina at Chapel Hill and North Carolina State University, Chapel Hill, NC 27599, USA; ksode@email.unc.edu

* Correspondence: tsugawa@cc.tuat.ac.jp; Tel.: +81-42-388-7027

Abstract

Nicotinoproteins are a group of NAD⁺-dependent dehydrogenases that bind NAD⁺ tightly and catalyze reactions without using free NAD⁺. In this study, we investigated the role of the unique insertion loop in nicotinoproteins. Carveol dehydrogenase (CADh), a short-chain dehydrogenase/reductase (SDR) nicotinoprotein, and β -hydroxybutyrate dehydrogenase from *Alcaligenes faecalis* (AfBHBDh), a non-nicotinoprotein counterpart, were used as model enzymes. An insertion loop-deleted mutant, CADh Δ 39–49, was constructed. An insertion loop from *Mycobacterium paratuberculosis* CADh (MpCADh) was introduced into AfBHBDh to generate the two mutants. The results showed that CADh Δ 39–49 lost NAD⁺ tight binding capacity and could not utilize free NAD⁺. In contrast, the AfBHBDh mutants showed no dye-mediated dehydrogenase activity. Moreover, the K_M and K_D values for NAD⁺ were higher than those of the wild-type enzyme. Docking simulations revealed a stronger binding affinity between NAD⁺ and the mutants than with the wild-type AfBHBDh. Taken together, these results suggest that the insertion loop interferes with NAD⁺ entry into the active site of the enzyme while creating a more energetically favorable binding environment. This loop is necessary but alone is insufficient to achieve NAD⁺ tight binding. This study deepens understanding of NAD⁺ binding in SDR nicotinoproteins and provides insights for SDR enzyme engineering.

Keywords: nicotinoprotein; short-chain dehydrogenase/reductase; protein engineering; NAD⁺-dependent dehydrogenase

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1. Introduction

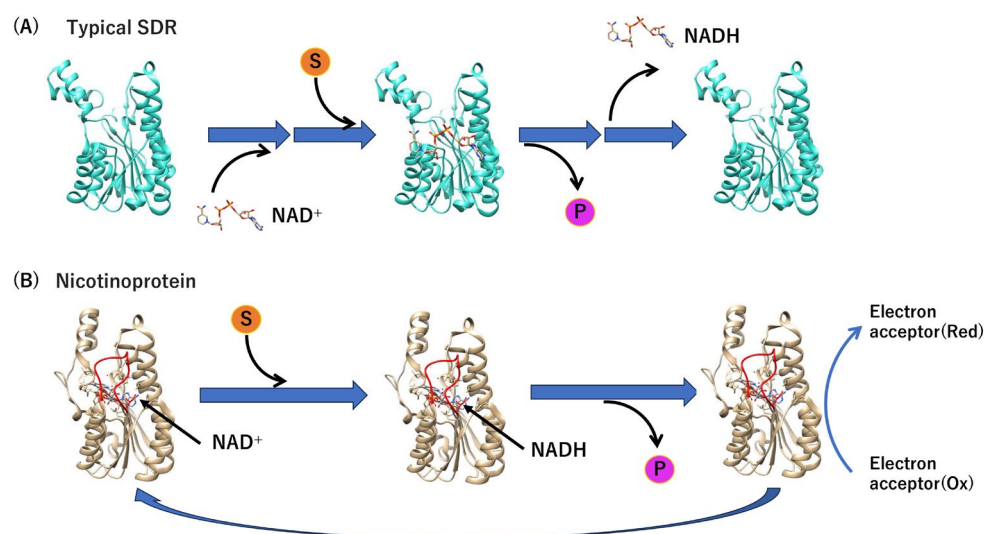
Nicotinamide adenine dinucleotide (NAD⁺)-dependent dehydrogenases (EC 1.1.1) are the most abundant class of oxidoreductases, accounting for approximately 90% of all oxidoreductases. They utilize NAD⁺ as the redox cofactor for substrate oxidation [1]. They

are classified into three major subfamilies based on their amino acid sequence lengths: long-chain, medium-chain, and short-chain dehydrogenases/reductases (SDRs) [1–6].

Typical NAD⁺-dependent dehydrogenases require free NAD⁺ as the redox cofactor for catalysis. External NAD⁺ binds transiently to the active site of the enzyme; substrate oxidation occurs concomitantly with NAD⁺ reduction to NADH (reduced nicotinamide adenine dinucleotide), and both NADH and the product rapidly dissociate from the enzyme. Thus, in typical NAD⁺-dependent dehydrogenases, enzyme–NAD⁺ complex formation represents only a transient association, requiring the continuous exchange of NAD⁺ from the external solution for catalytic turnover. Although NAD⁺-dependent dehydrogenases are commonly used for enzyme assays, their cofactor dependency creates critical challenges in biosensor development. Free NAD⁺ is expensive and unstable in a solution form, compromising its long-term storage stability. Moreover, direct NADH oxidation at electrodes requires high overpotentials, leading to NAD⁺ dimer formation and electrode fouling [7–10]. Even when artificial electron mediators such as phenanthroline dione, thionine, or 1,7-phenanthroline quinone are employed [7,11–13], the fundamental requirement for continuous NAD⁺ supplementation persists [14–20]. Both detection methods, direct NADH oxidation [21,22] and artificial electron mediator-assisted transfer, suffer from these inherent limitations, thereby significantly compromising the sensor design, operational costs, and reliability for continuous monitoring applications.

A minor group of NAD⁺-dependent dehydrogenases harboring non-exchangeable NAD⁺ within their structures has been reported. These enzymes are known as “nicotinoproteins” (Scheme 1). Unlike typical NAD⁺-dependent dehydrogenases, which require external NAD⁺ supplementation, nicotinoproteins contain internal NAD⁺ that is bound non-covalently but tightly within the structure and is not exchanged with free NAD⁺ in the solution. Instead of releasing NADH into the solution, nicotinoproteins utilize external electron acceptors such as 2,6-Dichloroindophenol (DCIP), p-nitrosoaniline, and N-nitrosodimethylamine to reoxidize internal NADH to NAD⁺, enabling continuous catalytic turnover without requiring external NAD⁺. Since their first report by Duine and coworkers in the 1990s, several nicotinoproteins have been identified and characterized, including formaldehyde dehydrogenase from *Pseudomonas putida*, alcohol dehydrogenase from *Amycolatopsis methanolica*, and carveol dehydrogenase from *Rhodococcus* and mycobacterial species [2,23–30]. Remarkably, these nicotinoproteins retain common structural preferences of typical NAD⁺-dependent dehydrogenases, including a conserved Rossmann fold for NAD⁺ binding [25]. Despite this structural conservation, one key structural feature that enables nicotinoproteins to harbor NAD⁺ internally is the presence of a distinctive “insertion loop” consisting of 10–20 amino acid residues (Figure 1). Structural analyses of nicotinoproteins have demonstrated that this insertion loop shields the internal NAD⁺ from the solvent and prevents cofactor exchange [2,25]. These studies suggest that this loop region is responsible for tight cofactor binding of nicotinoprotein dehydrogenases.

Nicotinoproteins can be conveniently used in biosensing because no free NAD⁺ is required in the turnover process. However, the number of reported nicotinoproteins remains limited. The development of a biosensor to create an artificial nicotinoprotein from a typical NAD⁺-dependent dehydrogenase is an alternative approach. Creating artificial nicotinoproteins and utilizing them as sensor recognition elements can become more cost-effective and easier, owing to the absence of unfavorable free NAD⁺. However, there is no clear explanation as to why nicotinoproteins can bind NAD⁺ tightly, and there is no established method for creating artificial nicotinoproteins. To establish a method for creating artificial nicotinoproteins, it is important to study the reason for the NAD⁺ tight binding of nicotinoproteins.



Scheme 1. Redox reaction pattern of a typical SDR and a nicotinoprotein SDR. The three-dimensional structures are shown in the ribbon view. (A) A typical SDR (*Af*BHBDh) which requires free and external NAD⁺ during the reaction in the solution. (B) A nicotinoprotein SDR (*Mp*CADh), in which the internal tightly bound NADH is oxidized by the external electron acceptor. The orange circle represents the substrate, and the purple circle represents the product.

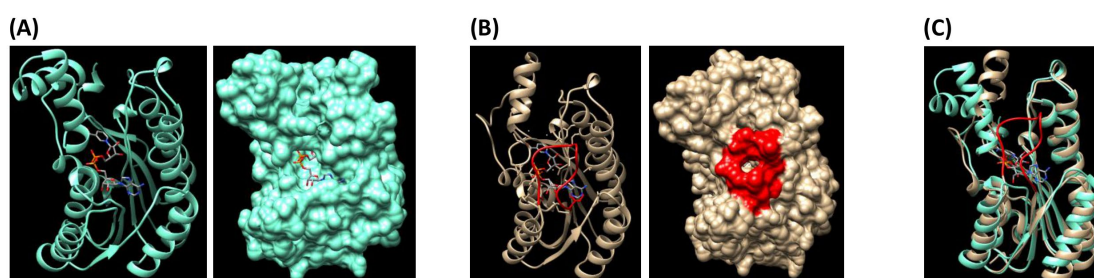


Figure 1. Ribbon and surface views of (A) *Af*BHBDh (PDB ID: 5B4T) and (B) *Mp*CADh (PDB ID: 3PGX) three-dimensional structures. (C) Overlapped ribbon structures of *Af*BHBDh and *Mp*CADh. The insertion loop is shown in red. NAD⁺ is shown in sticks and colored by element.

To elucidate the structural mechanisms underlying NAD⁺ tight binding in nicotinoproteins, this study focused on the insertion loop as a key structural determinant of NAD⁺ tight binding and adopted a protein engineering approach to experimentally demonstrate its functional role through two complementary approaches: deletion from the nicotinoprotein and introduction into a typical SDR. Carveol dehydrogenase (CADh), a nicotinoprotein belonging to the short-chain dehydrogenase/reductase (SDR) family, which is structurally conserved and facilitates direct comparison between nicotinoproteins and conventional SDRs, was selected. More specifically, complementary loss-of-function and gain-of-function approaches by creating an insertion loop-deleted *Re*CADh mutant (CADh Δ 39–49) and introducing the *Re*CADh insertion loop into β -hydroxybutyrate dehydrogenase derived from *Alcaligenes faecalis* (*Af*BHBDh), as a representative, well-characterized conventional SDR, were employed. The cofactor binding and enzymatic properties were evaluated using enzymatic activity measurements, surface plasmon resonance, and fluorescence spectroscopy. Additionally, *in silico* molecular modeling and docking simulations provided insights into structural changes and binding energetics. By investigating the necessity and sufficiency of the insertion

loops for cofactor binding, this study advances our fundamental understanding of nicotinoprotein mechanisms. Our findings could support the rational design of artificial nicotinoproteins for biotechnological applications.

2. Results

2.1. Design of CADh and BHBDh Mutants by Multiple Alignment

In the present study, CADhs were isolated from two species: *Rhodococcus erythropolis* DCL14 (ReCADh) and *Mycobacterium paratuberculosis* (MpCADh). These carved dehydrogenases, along with AfBHBDh as a representative typical SDR and five other conventional SDRs, were subjected to multiple sequence alignment (Figure 2). Both CADhs contain a characteristic insertion loop that distinguishes nicotinoproteins from typical SDRs. This region is located between the second β -sheet and the second α -helix, forming part of the NAD⁺-binding domain. The crystal structure analyzed by Haft et al. [25] demonstrated that in the tertiary structure of MpCADh, this insertion loop region restricts NAD⁺ accessibility to the solvent. Indeed, while typical SDRs exhibit an average NAD⁺ solvent-accessible surface area (SASA) of 82 Å², MpCADh shows only 18 Å² on average, suggesting that the insertion loop is a key structural determinant contributing to NAD⁺ tight binding.

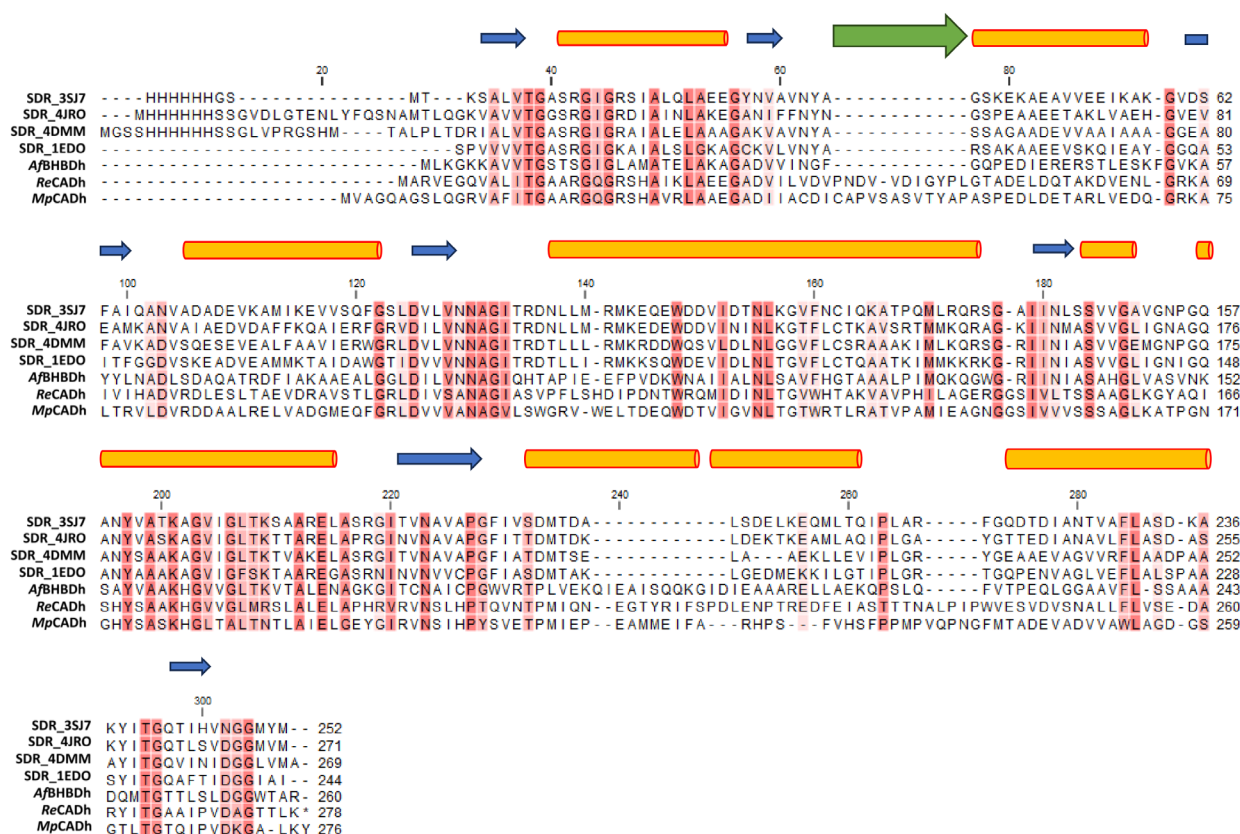


Figure 2. Multiple alignment of BHBDh, CADhs, and other typical SDRs. The β -sheet, α -helix, and the insertion loop of CADhs are shown as blue arrows, yellow syringes, and a green arrow, respectively. SDR_3SJ7: beta-ketoacetyl-CoA reductase (FabG) from *Staphylococcus aureus*. SDR_4JRO: 3-oxoacyl-[acyl-carrier-protein] reductase (FabG) from *Listeria monocytogenes*. SDR_4DMM: 3-oxoacyl-[acyl-carrier-protein] reductase from *Synechococcus elongatus* PCC 7942. AfBHBDh: β -hydroxybutyrate dehydrogenase from *Alcaligenes faecalis*. SDR_1EDO: *Brassica napus* beta-keto acyl carrier protein reductase. ReCADh (accession: Q9RA05.1): CADh from *Rhodococcus erythropolis* DCL14. MpCADh (PDB ID: 3PGX): CADh from *Mycobacterium paratuberculosis*. Residues were shaded according to sequence conservation. Positions with 70–100% sequence identity are

shown using a color gradient, with darker shading indicating higher conservation. The asterisk indicates the translation termination site.

For the loop deletion study, the loop-deleted mutant of *ReCADh* with the deletion of residues 39–49 (*CADh* Δ 39–49), which corresponds to the insertion loop region identified in *ReCADh*, was designed. For the study of loop insertion into a typical SDR, the design of the loop-inserted mutant was based on the sequence alignment of *AfBHBDh* with *MpCADh*. Alignment revealed that *MpCADh* contains a 12-residue insertion loop region (CAPVSVSVTYAP) at residues 44–55, corresponding to residues 36–37 of *AfBHBDh*. Furthermore, a comparison of the crystal structures of the *AfBHBDh* complex with NAD⁺ (PDB ID: 5B4T) and *MpCADh* (PDB ID: 3PGX) revealed that, in *AfBHBDh*, the NAD⁺ molecule from the adenine moiety to the phosphate group was exposed to the solvent, whereas in *MpCADh*, the NAD⁺ molecule was almost completely covered by the loop. Because insertion of the *MpCADh*-derived loop sequence into the corresponding site of *AfBHBDh* contributes to NAD⁺ tight binding, we designed loop-inserted mutants of *AfBHBDh* by inserting the *MpCADh*-derived loop sequence between Phe36 and Gly37 of *AfBHBDh*.

Additionally, to minimize structural perturbation caused by loop insertion, some conservative substitutions were introduced in the vicinity of the inserted loop to maintain local sequence compatibility. The insertion sites Phe36 and Gly37 correspond to Ile43 and Ala54 of *MpCADh*, respectively, and the BHBDh36–48 Cins mutant was designed, which includes the Ile substitution of Phe36 in *AfBHBDh* (Figure 3). Furthermore, the BHBDh33–50 Cins mutant was created, in which residues at both ends of the loop were mutated to the corresponding amino acid residues of *MpCADh* (N-terminal region of the insertion loop: Ile33Ala, Asn34Cys, Gly35Asp; C-terminal region of the insertion loop: Gly49Ala, Ser50Gln) (Figure 3).

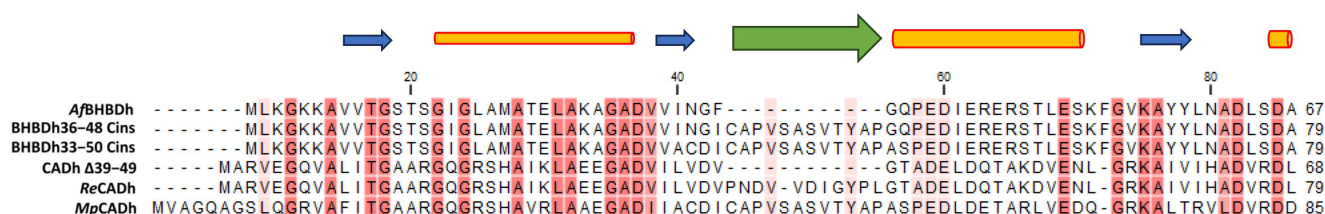


Figure 3. Alignment of the amino acid sequences around the insertion loop. *AfBHBDh*: BHBDh from *Alcaligenes faecalis*. *MpCADh*: CADh from *Mycobacterium paratuberculosis*. *ReCADh*: CADh from *Rhodococcus erythropolis* DCL14. The β -sheet, α -helix, and the insertion loop of CADhs are shown as blue arrows, yellow syringes, and a green arrow, respectively. Residues were shaded according to sequence conservation. Positions with 70–100% sequence identity are shown using a color gradient, with darker shading indicating higher conservation.

2.2. Evaluation of the Feature of the Insertion Loop-Deleted CADh Mutant

Nicotinoproteins exhibit enzymatic activity by utilizing artificial electron acceptors, such as DCIP, without the addition of free NAD⁺. To evaluate the effect of the loop deletion on the NAD⁺ retention capacity in nicotinoproteins, recombinant wild-type *ReCADh* and *CADh* Δ 39–49 were prepared. While the wild-type *ReCADh* was expressed as a soluble molecule, the *CADh* Δ 39–49 mutant was expressed as inclusion bodies. Therefore, both molecules were prepared from inclusion bodies using refolding procedures for further evaluation. Dehydrogenase activity in the absence of free NAD⁺ was evaluated using DCIP as an electron acceptor and was compared with that of native

ReCADh (prepared from the soluble fraction of the cell-free extract) and refolded *ReCADh* against 50 mM carveol.

The refolded *ReCADh* demonstrated dye-mediated dehydrogenase activity of $0.32 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, indicating reconstitution of NAD^+ into the enzyme during the refolding process. The refolded *CADh* $\Delta 39\text{--}49$ did not show dye-mediated dehydrogenase activity in the absence of free NAD^+ . No dye-mediated dehydrogenase activity was observed, even with free NAD^+ addition. The complete loss of catalytic function was confirmed in the presence and absence of NAD^+ .

To evaluate the retention of NADH within the enzyme molecule, fluorescence spectroscopy was performed (Figure 4). *ReCADh* showed emission peaks at 430 nm (excitation wavelength = 340 nm), which is characteristic of NADH in both native and refolded forms, indicating that NADH was retained within the *ReCADh* structure in both the native and refolded forms. On the other hand, *CADh* $\Delta 39\text{--}49$ did not show an emission peak at 430 nm (excitation wavelength = 340 nm), indicating that *CADh* $\Delta 39\text{--}49$ did not contain internal NADH (Figure 4A) and lost the capacity to bind NAD^+ tightly. These results clearly demonstrated that the insertion loop is essential for nicotinoprotein function.

Circular dichroism (CD) spectral analysis of the *CADh*s was performed, as shown in Supplementary Figure S1. *ReCADh* and *CADh* $\Delta 39\text{--}49$ showed approximately similar molar ellipticity after refolding, indicating that the secondary structure contents of *CADh* $\Delta 39\text{--}49$ were retained and not destroyed by loop deletion. Therefore, the loss of the dye-mediated activity was caused by the absence of the loop.

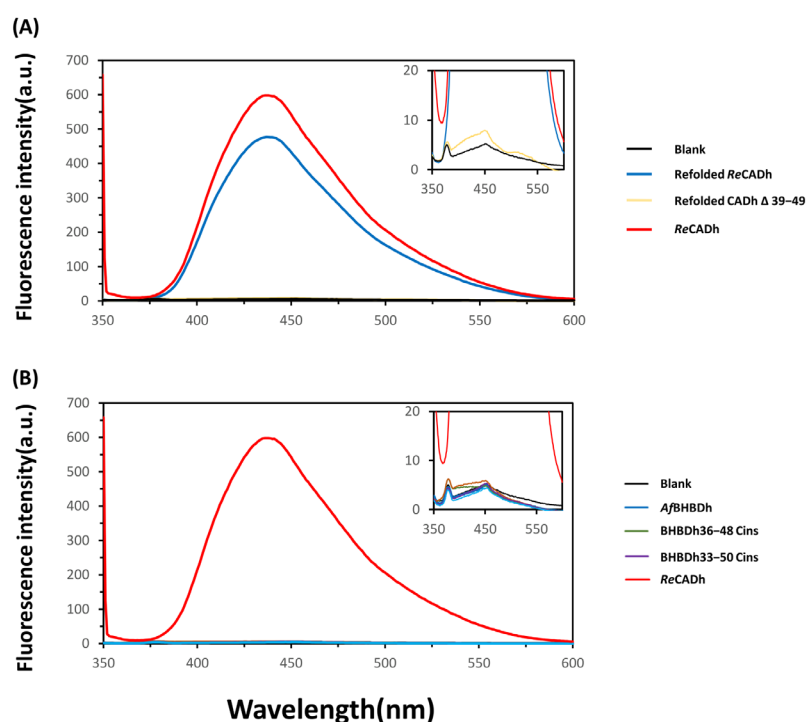


Figure 4. Fluorescence emission spectra of intrinsic NADH in BHBDhs and CADhs. (A) The emission spectra of 3.3 μM of *ReCADh*, refolded *ReCADh*, and refolded *CADh* $\Delta 39\text{--}49$ mutant. (B) The emission spectra of 3.3 μM of *A β BHBDh*, *BHBDh*36–48 Cins and *BHBDh*33–50 Cins and *ReCADh*. A magnified inset is shown within each panel to highlight the overlapping regions of the curves. The excitation wavelength was 340 nm.

2.3. Evaluation of Nicotinoprotein Activity and Existence of Internal NADH of Loop-Introduced BHBDh Mutants

After establishing the necessity of the insertion loop for nicotinoprotein function, the sufficiency of the insertion loop was investigated. To assess whether typical SDR mutants with an insertion loop derived from nicotinoproteins could acquire NAD⁺ tight-binding properties, wild-type BHBDh, BHBDh36–48 Cins, and BHBDh33–50 Cins mutants were prepared.

For BHBDh36–48 Cins and BHBDh33–50 Cins mutants, dye-mediated dehydrogenase activity against 40 mM (R)-(-)-3-Hydroxybutyric acid sodium (R)-(-)BHB) was evaluated in the absence of free NAD⁺. However, no apparent activity was observed. In addition, in the fluorescence spectrum, the mutants with neither loop insertion showed an emission peak at 430 nm (Figure 4B). Although the reconstitution of NAD⁺ with these mutants was attempted through refolding procedures, no apparent dye-mediated activity was observed in the refolded samples. Therefore, the loop-inserted mutants did not retain NAD⁺ internally and lacked the capacity of NAD⁺ tight binding.

2.4. Enzyme Kinetics of BHBDh Mutants and the Interaction Between the BHBDh Mutants and NAD⁺

To elucidate the mechanisms underlying the lack of dye-mediated dehydrogenase activity in the loop-inserted mutants, the effects of the insertion loop on the interaction between external NAD⁺ and the enzyme were investigated. BHBDh activity in the presence of free NAD⁺ was measured by monitoring NADH production at 340 nm (Tables 1 and 2). The substrate (R)-(-)BHB concentration dependence is shown in Table 1 and Supplementary Materials, Figure S2.

Unexpectedly, the loop-inserted mutants showed BHBDh activity in the presence of external NAD⁺. Previous studies demonstrated that *Mp*CADh cannot exchange NAD⁺ within a molecule with NAD⁺ in the solvent, as verified by STD-NMR [25]. However, the mutants were still able to utilize the external NAD⁺ in the solvent for catalysis. When comparing $V_{\max}/K_M$ values, all mutants showed lower $V_{\max}/K_M$ values than the wild-type *A/BHBDh* (Table 1). BHBDh36–48 Cins showed a 4.6-fold decrease in $V_{\max}/K_M$ compared with *A/BHBDh*, whereas BHBDh33–50 Cins showed a 53-fold decrease compared with *A/BHBDh*.

Table 1. Kinetic parameters of BHBDhs toward (R)-(-)-BHB determined from NADH formation assays.

Enzyme	$V_{\max}$ ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	K_M for (R)-(-)BHB (mM)	$V_{\max}/K_M$ ($\text{mM}^{-1}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)
<i>A/BHBDh</i>	841 ± 67.3	5.08 ± 0.55	166
BHBDh36–48 Cins	769 ± 116	21.4 ± 3.03	35.9
BHBDh33–50 Cins	55.7 ± 2.45	17.9 ± 3.28	3.11

NADH formation monitored at 340 nm in the presence of 20 mM NAD⁺.

To examine the NAD⁺-exchangeable properties retained by the loop-inserted mutants, the NAD⁺ concentration dependency of BHBDh activity with a racemic 3-Hydroxybutyric acid (racemic BHB) concentration of 80 mM was evaluated (Table 2 and Supplementary Materials, Figure S3). Compared with *A/BHBDh*, BHBDh36–48 Cins and BHBDh33–50 Cins showed 18-fold and 79-fold increases in K_M for NAD⁺, respectively. The $V_{\max}$ value of BHBDh33–50 Cins decreased by 7-fold, and the $V_{\max}/K_M$ value of BHBDh36–48 Cins for NAD⁺ was 16-fold lower than that of *A/BHBDh*. The $V_{\max}/K_M$ values of BHBDh33–50 Cins were 552-fold lower than those of *A/BHBDh*, which was caused by a significant decrease in $V_{\max}$ and a significant increase in K_M . The $V_{\max}/K_M$ for (R)-(-)BHB

decreased by 4.6-fold and 53-fold in BHBDh36–48 Cins and BHBDh33–50 Cins, respectively. The decrease in $V_{\max}/K_M$ for NAD^+ was significantly more pronounced. This suggests that the differences in $V_{\max}/K_M$ values for BHB or NAD^+ in the loop-inserted mutants may be due to the greater effects on NAD^+ binding rather than on substrate binding. Specifically, the decreased BHBDh activity in the mutants may be attributed to the effects on NAD^+ exchange rather than substrate binding, particularly due to steric hindrance caused by the nicotinoprotein-derived loop, leading to restricted NAD^+ availability.

A/BHBDh and BHBDh36–48 Cins showed similar $V_{\max}$ values but significantly different K_M values against NAD^+ . Since this difference was reflected in the affinity between NAD^+ and each enzyme, surface plasmon resonance (SPR) measurements were conducted to analyze the binding affinity of each enzyme to NAD^+ . The SPR sensorgram results are shown in the Supplementary Materials, Figure S4. A/BHBDh and BHBDh36–48 Cins showed NAD^+ concentration-dependent signal increases, a typical pattern of enzyme– NAD^+ interactions in NAD^+ -dependent dehydrogenases. The association and dissociation rates of NAD^+ were too rapid to reliably determine k_{on} and k_{off} . Based on the equilibrium assay, the K_D value of A/BHBDh was calculated as 1.3×10^{-6} M, while that of BHBDh36–48 Cins was 8.7×10^{-6} M, which was 6-fold higher than that of the wild type. The increased K_D value in the mutant suggests a reduced binding affinity for NAD^+ , indicating that the decreased NAD^+ availability in the mutant may be attributed to the inhibition of NAD^+ binding by the insertion loop.

Table 2. Kinetic parameters of BHBDhs toward NAD^+ determined from NADH formation assays. NADH formation monitored at 340 nm in the presence of 80 mM racemic BHB.

Enzyme	$V_{\max}$ ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	K_M for NAD^+ (mM)	$V_{\max}/K_M$ ($\text{mM}^{-1}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)
A/BHBDh	271 ± 21.5	0.14 ± 0.09	1954
BHBDh36–48 Cins	304 ± 33.5	2.52 ± 0.70	121
BHBDh33–50 Cins	38.6 ± 3.00	11.0 ± 0.65	3.52

Thermal stability was measured to evaluate the effects of mutations on enzyme stability. The results are shown in the Supplementary Materials, Figure S5. The mutants were slightly less stable than A/BHBDh; however, this difference was not statistically significant. Therefore, the effects on enzymatic activity characteristics were largely attributed to the effects of the mutation rather than to the loss of activity during the experiments.

2.5. In Silico Characterization and Comparison of BHBDhs and ReCADhs

Docking simulations between the enzyme molecule and NAD^+ were repeated 20 times per sample using the AutoDock Vina 1.1.2 software (Figure 5). The structure used in the docking simulation was predicted using AlphaFold 3 software (Supplementary Materials, Figure S6). A/BHBDh and NAD^+ showed an average binding affinity of -8.91 $\text{kcal}\cdot\text{mol}^{-1}$ (± 0.84 $\text{kcal}\cdot\text{mol}^{-1}$), indicating a typical range of binding free energy between the enzyme and cofactor. BHBDh36–48 Cins and BHBDh33–50 Cins showed -10.5 $\text{kcal}\cdot\text{mol}^{-1}$ (± 0.73 $\text{kcal}\cdot\text{mol}^{-1}$) and -10.7 $\text{kcal}\cdot\text{mol}^{-1}$ (± 0.27 $\text{kcal}\cdot\text{mol}^{-1}$) binding free energy with NAD^+ , respectively. The docked NAD^+ of the mutants was located at the same position as in the structure of the docked crystal structure of A/BHBDh, the NAD^+ binding site of the enzyme. These results indicated that the mutants bound to NAD^+ at the binding site of the enzyme with stronger affinity than A/BHBDh in a static scenario. Furthermore, a docking simulation of ReCADh, which contains no NAD^+ , was performed; NAD^+ did not dock in the same position or orientation as observed for the crystal structure of MpCADh. The internal NAD^+ in the crystal structure of ReCADh (PDB ID: 3PGX) suggested that free

NAD⁺ might not have bound to *Re*CADh at its binding site. The solvent-accessible surface area of NAD⁺ in the mutated protein, as predicted using MODELLER 10.1, was calculated. Modeling was necessary because AlphaFold 3 did not predict the closed conformation, although the crystal structure of BHBDhs complexed with NAD (closed form) has been reported (PDB ID: 5B4T) [31]. The SASA of NAD⁺ in the predicted mutant structures was then compared with that of NAD⁺ in the crystal structures of the wild-type proteins. The results (Table 3) showed that *Af*BHBDh has an NAD⁺ SASA of 94.2 Å², while the mutants and CADhs showed that the SASA was 25.0–11.2 Å². This result indicates that the introduction of an insertion loop decreased the SASA of NAD⁺, resulting in increased affinity between the enzyme and NAD⁺.

The results of molecular dynamics simulation of BHBDhs and *Re*CADhs are shown in the Supplementary Materials, Figure S7. The Root Mean Square Fluctuation (RMSF) value of the insertion loop (residues 36–50: highlighted in yellow in Supplementary Materials, Figure S7B,D) of the BHBDh mutants was higher than that of CADhs, indicating that the artificially inserted loop was less stable than the natural insertion loop. Nevertheless, BHBDh33–50 Cins exhibited decreased flexibility in the insertion loop and substrate-binding domain (residues 184–239: highlighted in green in Supplementary Materials, Figure S7B,D). This behavior is similar to that of CADhs, suggesting that BHBDh33–50 Cins shares structural characteristics with CADhs when NAD⁺ is present.

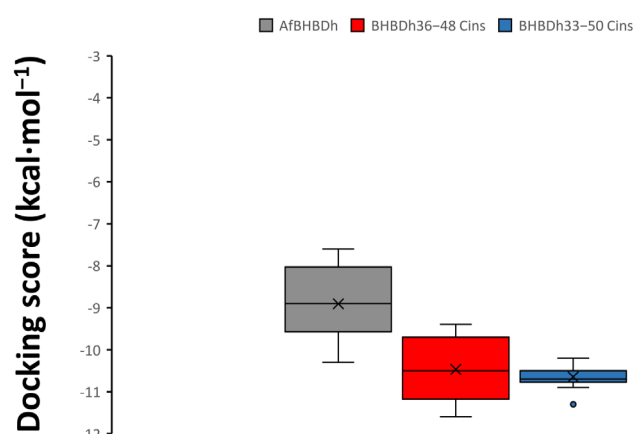


Figure 5. The binding free energy between NAD⁺ and the enzyme, determined by docking simulation using AutoDock Vina software. The cross indicates the centroid of the projected docking score distribution. The square region represents one standard deviation from the mean along each projected axis, reflecting the dispersion of docking scores in the projected space. The outer boundary corresponds to the full range in which docking poses were observed.

Table 3. Comparison of BHBDhs and CADhs cofactor surface area and solvent-accessible surface area.

Enzyme	Structure Source Protein	Surface Area (Å ²)	Cofactor Total Surface Area (Å ²)	Cofactor Solvent-Accessible Surface Area (Å ²)
<i>Af</i> BHBDh	PDB ID: 5B4T	11,429	824	94.2
BHBDh36–48 Cins	MODELLER	10,868	949	25.0
BHBDh33–50 Cins	MODELLER	10,871	949	13.4
<i>Mp</i> CADh	PDB ID: 3PGX	10,976	818	19.4
<i>Re</i> CADh	MODELLER	11,742	817	11.2

3. Discussion

This study focused on the unique “insertion loop” of nicotinoproteins, which shields the adenine part of NAD⁺ and is a common structure among nicotinoproteins. The role of this unique insertion loop of nicotinoproteins has been discussed in studies on medium-chain dehydrogenase/reductase (MDR) enzymes, such as alcohol dehydrogenase and formaldehyde dehydrogenase, and short-chain dehydrogenase/reductase (SDR) enzymes, such as CADh. The NAD⁺ tight binding to nicotinoprotein is thought to be facilitated by the insertion loop, which reduces the SASA of internal NAD⁺ [2,25]. However, protein engineering approaches targeting the insertion loop of nicotinoproteins have not been systematically investigated.

This paper presents two protein engineering approaches that focus on the insertion loop. First, the insertion loop of nicotinoproteins was deleted, and the nicotinoprotein Δ loop mutant was generated, which lost NAD⁺ tight binding, confirming the essential role of the insertion loop. Second, the loop was inserted into a typical SDR *A/BHBDh*, yielding two mutants: BHBDh36–48 Cins and BHBDh33–50 Cins. The functional properties of these mutants were evaluated. The results showed that typical SDR mutants did not acquire the desired NAD⁺ tight binding features. Specifically, the fluorescence spectra of the loop-inserted mutants lacked a specific NADH peak, and the mutants maintained the ability to exchange NAD⁺ with external NAD⁺ during catalytic cycles. In contrast, in nicotinoproteins, internal NAD⁺ is non-exchangeable [25–30]. Overall, these results demonstrate that introducing the insertion loop did not confer NAD⁺ tight binding in the mutants.

The SASA of the internal NAD⁺ of the insertion loop-inserted mutants decreased. In addition, by introducing the insertion loop, the average binding affinity (binding Gibbs free energy) between the insertion loop-inserted mutants and internal NAD⁺ decreased, as shown by the docking simulation (Figure 5). This suggests that under static conditions, the protein environment of the NAD-bound enzyme of the loop-inserted mutants became more favorable for binding NAD than the wild-type enzyme by lowering the SASA. This supports the hypothesis that reduced solvent accessibility enhances binding between the enzyme and NAD⁺ via hydrophobic effects [25,32,33].

Two contradictory results were shown: 1. SPR showed that the mutant had a higher K_D value, indicating a lower affinity for free NAD⁺ than *A/BHBDh*. 2. Docking simulations showed that the mutant had a lower binding Gibbs free energy, indicating that the mutant had a higher affinity for free NAD⁺ than the *A/BHBDh*. These results can be explained by the proposed mechanism that the insertion loop of the BHBDh mutant acts like a cap that blocks free NAD⁺ from entering the active site by steric hindrance, resulting in lower affinity toward free NAD⁺ and higher K_D values. Although the enzyme does not bind free NAD⁺ in the solution, once NAD⁺ is bound internally, the loop locks it in place and increases hydrophobic interactions by reducing its exposure to the solvent, resulting in higher affinity than the *A/BHBDh* in docking simulations [32,33]. These structural features of the loop settle the experimental and computational findings, reconfirming the finding that nicotinoproteins tightly bind their internal NAD⁺ while it is non-exchangeable with free NAD⁺. These observations indicate that an insertion loop is necessary but not sufficient for NAD⁺ tight binding. In other words, it is reasonable to assume that there are factors other than the insertion loop that achieve the NAD⁺ tight binding. One possibility is the significant structural differences in the substrate-binding domain between typical SDR and CADh, which may contribute to their functional differences and are considered another factor for NAD⁺ tight binding. In typical SDRs, the substrate-binding domain moves toward the active site, and this movement has been suggested to be crucial for enzyme activity [34–40]. In particular, the substrate-binding domain of BHBDh (residues 191–219, described as ‘small domain’ by Kanazawa et al. [31]) is known to exhibit

conformational variability, allowing the overall structure of the domain to undergo movement upon binding NAD⁺ or the substrate. This results in open or closed enzyme conformations.

In the open conformation, a cleft is formed between the substrate-binding domain and the main structure, allowing NAD⁺ to enter and exit the enzyme. Nevertheless, the crystal structure of BHBDh in the open conformation (PDB ID: 5B4T) showed that internal NAD⁺ was absent, whereas in the closed conformation (PDB ID: 2YZ7), NAD⁺ was complexed. To date, the structural changes in the substrate-binding domain of CADh upon substrate binding and release have not been studied in detail. In this study, CADh was assumed to have lower flexibility than typical SDRs, and it was difficult for it to undergo such a drastic conformational change, creating a protein intramolecular microenvironment favorable for cofactor binding. Therefore, both a less flexible substrate-binding domain and an insertion loop are required for NAD⁺ tight binding.

This is the first report that attempted to investigate the role of the unique insertion loop of the nicotinoprotein CADh, a NAD⁺ tight binding dehydrogenase. Although the loop-inserted mutants did not show the typical features of nicotinoproteins (tight binding with NAD⁺) and showed no dye mediation without using free NAD⁺, several insights were acquired in this study. (1) This is the first report to study the interaction, structure, and trajectory of the insertion loop of nicotinoproteins from an in silico perspective. (2) This report revealed that the insertion loop is necessary but not sufficient for NAD⁺ tight binding. (3) This study verified that free NAD⁺ access to the enzyme was interfered with by the insertion loop.

4. Materials and Methods

4.1. Chemicals and Materials

Glycine, sodium chloride (NaCl), magnesium sulfate (MgSO₄), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), Tween-80, Tween-20, and agarose S were purchased from Kanto Kagaku (Tokyo, Japan). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Dojindo Laboratories, Kumamoto, Japan), imidazole (TCI, Tokyo, Japan), nickel(II) chloride, and kanamycin sulfate were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). The yeast extract, tryptone, and NAD⁺ were purchased from Nacalai Tesque (Kyoto, Japan). (R)-(-)-3-Hydroxybutyric acid sodium and racemic sodium 3-hydroxybutyrate were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas 2,6-Dichloroindophenol sodium salt hydrate (DCIP) was purchased from Merck (Darmstadt, Germany). All the chemicals were of reagent grade.

4.2. Construction of Expression Vectors

The codon-optimized ReCADh was synthesized (DNA and amino acid sequences; see Supplementary Materials) and inserted into the pET-28a(+) vector (Merck, Darmstadt, Germany). The expression vector for the wild-type BHBDh from *Alcaligenes faecalis* NBRC 13111 was constructed as previously described [41]. Site-directed mutagenesis for AfBHBDh was performed using the QuikChange method with KOD-Plus-Neo (Toyobo, Osaka, Japan) and two primers (Supplementary Materials). The synthetic genes and primers were purchased from Eurofins Scientific SE (Luxembourg City, Luxembourg).

The extra 12 residues from the insertion loop of ReCADh were introduced into AfBHBDh by overlapping polymerase chain reaction (PCR) using two primer sets (Supplementary Materials). Additional 12 residues from the insertion loop of ReCADh were deleted by overlapping PCR using two primer sets (Supplementary Materials). The PCR product was ligated to pET-28a(+) using NdeI and HindIII restriction sites. The

vectors for the expression and the product of *Af*BHBDh with the insertion loop were named BHBDh36–48 Cins and BHBDh33–50 Cins, respectively. Based on BHBDh36–48 Cins, I33A, N34C, G35D, G49A, and S50Q mutations were introduced by infusion PCR, and the vector was named pET-28a(+) BHBDh33–50 Cins. The insertion loop-defected *Re*CADh mutant was called CADh Δ 39–49. Plasmid mutations were confirmed by sequencing.

4.3. Preparation of BHBDhs and CADh

LOBSTR BL21 (DE3) cells (Cosmo Bio, Tokyo, Japan) were transformed into *Af*BHBDh expression vectors. Transformed cells were cultured in 500 mL baffled flasks containing 100 mL ZYP-5052 medium [0.5% glycerol, 0.05% glucose, 0.2% lactose, 50 mM KH_2PO_4 , 25 mM $(\text{NH}_4)_2\text{SO}_4$, 50 mM Na_2HPO_4 , and 1 mM MgSO_4] containing 50 $\mu\text{g/mL}$ kanamycin for 48 h at 25 °C as the main culture. Cells were harvested at 5000 $\times$ g for 30 min at 4 °C.

Harvested cells were resuspended in 20 mM PPB containing 20 mM imidazole, 0.5 M NaCl, 10% glycerol, and 0.1% Tween-80 (pH of 7.0) and disrupted by French pressure. The homogenate was centrifuged at 5000 $\times$ g for 30 min at 4 °C. The supernatant was ultracentrifuged at 105,655 $\times$ g for 1 h at 4 °C, and the resulting supernatant was designated as the crude enzyme. The crude enzyme samples were applied to a HisTrap™ HP column (Cytiva, Marlborough, MA, USA) that had been equilibrated with 20 mM PPB (pH of 7.0) containing 500 mM NaCl, 20 mM imidazole, 10% glycerol, and 0.2% Tween-80 (Supplementary Materials, Figure S8). The absorbed proteins were eluted using a linear imidazole gradient (20–500 mM).

4.4. Refolding of CADh Δ 39–49

The harvested cells of BL21 LOBSTR (DE3) with CADh Δ 39–49 expression vectors were resuspended in 0.1 M PPB, 0.1 M NaCl, 1 mM DTT, and 1 mM EDTA, ultrasonically disintegrated for 30 s, and then rested for 30 s; this was done 30 times. Then the cell lysate was centrifuged at 20,000 $\times$ g at 4 °C for 20 min, and the pellet was collected. The pellet was vibrated and washed with 0.1 M PPB, 0.1 M NaCl, 1 mM DTT, 1 mM EDTA, and 0.5% Tween 20 at 1500 rpm for 30 min and centrifuged at 20,000 $\times$ g and 4 °C for 20 min three times, and the pellet was washed and collected. The washed pellet was then dissolved in 0.1 M PPB, 0.1 M NaCl, 1 mM DTT, 1 mM EDTA, and 6 M Urea at 1500 rpm for 3 h at 4 °C and centrifuged at 20,000 $\times$ g and 4 °C for 20 min. The supernatant was collected and diluted in 0.1 M PPB, 1 mM DTT, 10% glycerol, and 500 μM NAD^+ . The diluted supernatant was incubated at 10 °C for 90 h. The diluted supernatant was then concentrated by a Amicon® Ultra-0.5 Centrifugal Filter Unit (10 kDa MWCO) (Merck Millipore, Burlington, VT, USA) at 4000 rpm and 4 °C for 20 min, followed by dialysis in 20 mM PPB (pH of 7.0). Finally, the diluted supernatant was purified using Liquid Chromatography, as described in Section 4.3.

4.5. Enzyme Assay

NAD^+ -dependent BHBDh activity was determined by monitoring NADH formation at 340 nm ($\epsilon = 6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) using a Shimadzu UV mini 1240 (Shimadzu, Kyoto, Japan). The enzyme samples were incubated at 25 °C with 100 mM PPB (pH of 7.0) and various BHB and NAD^+ concentrations. K_M and $V_{\max}$ values as described in the Michaelis–Menten equation were calculated using the Hanes–Woelf plot.

The nicotinoprotein dye-mediated activity was determined in the absence of free NAD^+ . The enzyme sample was incubated at 25 °C with 100 mM PPB (pH of 7.0) and various (R)-(–)BHB concentrations as well as with 0.06 mM DCIP. The decrease in absorbance at 600 nm due to DCIP ($\epsilon = 22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) was monitored.

The thermal stability of each mutant was evaluated using a dehydrogenase activity assay (BHB: 40 mM; NAD⁺: 1 mM) after 10 min of incubation at each temperature in a water bath. Orange: AfBHBDh. Red: BHBDh36–48 Cins. Black: BHBDh33–50 Cins. Each experiment was conducted in triplicate. Error bars show the standard deviation.

4.6. Fluorescence Measurement

The fluorescence was measured using a JASCO FP-6500 spectrofluorometer (JASCO, Tokyo, Japan). The fluorescence emission spectra were measured between 350 and 600 nm at an excitation wavelength of 340 nm. The fluorescence excitation spectra were scanned between 250 and 400 nm at a fixed emission wavelength of 430 nm. The concentration of the enzyme solution was 0.1 mg mL⁻¹ in 20 mM PPB (pH of 7.0).

4.7. Modeling and Structure Prediction

The structures of NAD⁺-bound or -unbound ReCADh, BHBDh36–48, and BHBDh33–50 Cins were predicted using AlphaFold 3 Beta [42] with NAD⁺ as the ligand.

4.8. Docking Simulation

AutoDock Vina 1.1.2 was used to simulate the docking of proteins with NAD⁺ and to calculate their binding affinities. Docking simulation of AfBHBDh, BHBDh36–48 Cins, BHBDh33–50 Cins, ReCADh, and CADh Δ39–49 between NAD⁺ was performed by Autodock Vina as described by Trott and Olson [43].

Files for docking simulations were created using Python Molecule Viewer 1.5.6. First, hydrogen atoms were added to each model structure. The flexible regions were set as the inserted loop or its corresponding residues and as residues that could interact with NAD⁺. A ligand file was created using rotatable bonds for rotation during the simulation.

The size of the grid box was set to cover all structures of the protein, with NAD⁺ as the ligand. For the simulation, the Central Processing Unit, exhaustiveness, num_modes, and energy_range parameters were set to 8, 10, 100, and 3, respectively. The output files and structures were analyzed using PyMOL 1.0.0.0.

4.9. Circular Dichroism Measurement

Circular dichroism measurements were performed by using Jasco J-820 (JASCO, Tokyo, Japan) at 25 °C. For this, 300 μL of a 60 μg mL⁻¹ protein sample diluted with 20 mM PPB, at a pH of 7, was measured in a quartz cuvette with a 0.1 mM optical path length. The machine was then warmed and flushed with nitrogen gas for 1 h. The wavelength range was 250–190 nm. The scan speed was 30 nm min⁻¹, and scanning was performed 5 times. The data were processed using the Spectra Manager™ Ver. 2.

4.10. Molecular Dynamics Simulation

Molecular dynamics simulations were performed using GROMACS 2024 with a CHARMM36m force field. Protein–NAD⁺ complexes were placed in a triclinic box containing Simple Point Charge water molecules. For this, 0.01 M K₂HPO₄ and KH₂PO₄ were used as counterions to balance the charge in the simulation system and to mimic the physiological conditions of the system. A GROMACS force field was used. The system environment contained a protein complex, water molecules, and ions, which were briefly equilibrated before the production run. The time of the MD simulation was 200 ns, the system pH was 7.0, and the temperature was 300 K. GROMACS built-in packages were used to analyze the simulation trajectories, which were plotted using Excel 2016 MSO ver 2601.

5. Conclusions

A previous study suggested that the unique insertion loop of the SDR nicotinoprotein is the reason why nicotinoproteins can bind to NAD⁺ tightly and is non-exchangeable. Based on this hypothesis, on one hand, this study investigated the role of the unique insertion loop of nicotinoproteins experimentally, introduced an insertion loop of *ReCADh* into the non-nicotinoprotein BHBDh, and created mutants. However, these mutants did not convert into nicotinoproteins. The mutants did not bind NAD⁺ tightly and did not show nicotinoprotein dye-mediated activity with the artificial electron acceptor or without free external NAD⁺. Enzyme kinetics and SPR indicated that the insertion loop of the mutants interfered with the entry and exit of NAD⁺ from the protein. In contrast, the insertion defect in *ReCADh* resulted in the loss of its ability to bind with NAD⁺ tightly. Finally, in silico analysis verified the stability of the BHBDh mutants and suggested that the interaction between the protein and NAD⁺ was strengthened under static conditions. These results indicated that the insertion loop is a necessary condition but not the only factor for NAD⁺ tight binding of the nicotinoprotein, and the conformation of substrate-binding domain of the SDR might be another important factor. Further investigation is required to clarify this.

Although in the 2000s the screening and biochemical study of nicotinoproteins became popular, fundamental research on the interaction between NAD⁺ and nicotinoproteins was limited. This was due to a lack of information regarding the structure and biochemical properties of the insertion loop. Basic research should be performed to clarify the tight NAD⁺ binding mechanism of nicotinoproteins.

Supplementary Materials: The supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ijms27052367/s1>.

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