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Exploration of the Glycine Residues Essential for NAD^+-binding of *Bacillus licheniformis* Aldehyde Dehydrogenase

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Abstract. The NAD^+-requiring enzymes of the aldehyde dehydrogenase (ALDH) family contain a glycine motif, $GX_{1-2}GXXG$, which is reminiscent of the fingerprint region of the Rossman fold, a conserved structural motif of the classical nicotinamide nucleotide-binding proteins. In this research, the role of three glycine residues situated within the putative NAD^+-binding motif (211-GPGSSAG) together with Gly233 and Gly238 of *Bacillus licheniformis* ALDH (*Bl*ALDH) were probed by site-directed mutatgenesis. Fifteen mutant *Bl*ALDHs were obtained by substitution of the indicated glycine residues with alanine, glutamate and arginine. Except for the Ala replacement at positions 211, 213, 217 and 238, the remaining mutant enzymes lost the dehydrogenase activity completely. Tryptophan fluorescence and far-UV circular dichroism spectra allowed us to discriminate *Bl*ALDH and the inactive mutant enzymes, and unfolding analyses further revealed that they had a different sensitivity towards temperature- and guanidine hydrochloride (GdnHCl)-induced denaturation. *Bl*ALDH and the functional variants had a comparable *T*m value, but the value was reduced by more than 5.1°C in the rest of mutant enzymes. Native *Bl*ALDH started to unfold beyond ~ 0.21 M GdnHCl and reached an unfolded intermediate, $[GdnHCl]_{0.5,\ N\text{-}U}$, at 0.92 M equivalent to the free energy change ($\Delta G_{N\text{-}U}$) of 12.34 kcal/mol, whereas the denaturation midpoints for mutant enzymes were 0.45–1.61 M equivalent to $\Delta G_{N\text{-}U}$ of 0.31–4.35 kcal/mol. Taken together, these results strongly suggest that the explored glycines are indeed important for the catalytic activity and structural stability of *Bl*ALDH

Keywords: Aldehyde dehydrogenase, NAD^+-binding motif, mutagenesis, thermal unfolding, guanidine hydrochloride

1. Introduction

Bacillus licheniformis is a Gram-positive, spore-forming soil bacterium that has long been employed as a microbial cell factory for the commercial production of exoenzymes. In the genome of this bacterium, more than 10 ALDH genes have been identified [1], and one of these genes, ycbD, encodes an ALDH enzyme comprising of 488 amino acid residues. To understand the structure-function relationship of the cloned enzyme, the directed evolution strategy is pursued to verify the catalytic role of the conserved Glu255 and Cys289 residues [2]. Beyond these observations, it is worth noting that the recombinant enzyme prefers NAD^+ as a cofactor in the oxidation reaction of aliphatic aldehydes [3]. In nicotinamide dinucleotide binding proteins, a glycine-rich phosphate-binding loop has been shown to connect the C-terminus of β1 with the N-terminus of αA [4]. To our knowledge, the importance of conserved glycine residues in the corresponding motif of *Bl*ALDH has not been studied yet (Fig. 1a). By performing the computer modeling with the template of *Pseudomonas aeruginosa* betaine aldehyde dehydrogenase (PDB code: 2wme), two other conserved glycine residues, Gly233 and Gly238, were found to be located at the proximity positions of the cofactor (Fig. 1b). The situation of these two residues in the putative active site of *Bl*ALDH was warranted their exploration as the essential residues for the enzyme.

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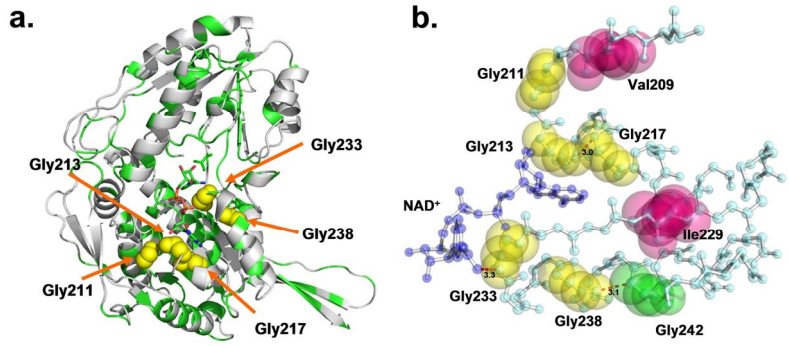

Fig. 1

2. Methods

2.1. Dehydrogenase activity assay

The dehydrogenase activity was calculated from the reduction of NAD^+ to NADH at 340 nm, and the amount of NADH formed was determined with $\Delta\varepsilon_{340}$ = 6.22/mM/cm. One unit of *Bl*ALDH was defined as the amount of enzyme that reduced 1 µmol of NAD^+ to NADH per minute. Values of K_M and $\boldsymbol{k}_{cat}$ were determined by fitting the initial rates as a function of substrate concentration to the Michaelis-Menten equation.

2.2. Circular dichroism (CD)

All far-UV CD spectra were recorded by a JASCO model J-815 equipped with Peltier thermostated cuvette holder under constant nitrogen flow. The data were expressed as molar ellipticity (deg · cm^2 · $dmol^{-1}$) based on a residue number of 488 and a mean molecular weight (MRW) of 52.7 kDa. Molar ellipticity was calculated as $[\theta]$ = [100 x (MRW) x $\theta_{obs}/(c \times l)$], where θ_{obs} is the observed ellipticity in degree at a given wavelength, c is the protein concentration in mg/ml, and l is the length of the light path in cm.

Thermal denaturation of the parental and mutant enzymes was followed by continuously monitoring ellipticity changes at a fixed wavelength of 222 nm over the temperature range of 20–90°C. The unfolding data of *Bl*ALDH and its derivatives were treated with the thermodynamic model by global fitting of the data [5]. The two-state unfolding model (Scheme I) was described by Eq. 1.

$$N \xleftrightarrow{K_{N-U}} U$$

(Scheme I)

$$\theta_{222} = \frac{\theta_N + \theta_U \cdot \exp\left[-\frac{\Delta \boldsymbol{H}_U}{\boldsymbol{RT}} \cdot \left(1 - \frac{\boldsymbol{T}}{\boldsymbol{T}_m}\right) + \frac{\Delta \boldsymbol{C}_{PU}}{\boldsymbol{RT}} \cdot \left(\boldsymbol{T} \ln\left(\frac{\boldsymbol{T}}{\boldsymbol{T}_m}\right) + \boldsymbol{T}_m - \boldsymbol{T}\right)\right]}{1 + \exp\left[-\frac{\Delta \boldsymbol{H}_U}{\boldsymbol{RT}} \cdot \left(1 - \frac{\boldsymbol{T}}{\boldsymbol{T}_m}\right) + \frac{\Delta \boldsymbol{C}_{PU}}{\boldsymbol{RT}} \cdot \left(\boldsymbol{T} \ln\left(\frac{\boldsymbol{T}}{\boldsymbol{T}_m}\right) + \boldsymbol{T}_m - \boldsymbol{T}\right)\right]} \quad (1)$$

where $\boldsymbol{\theta}_{222}$ is the relative ellipticity at 222 nm, $\boldsymbol{\theta}_N$ and $\boldsymbol{\theta}_U$ are the calculated ellipticities of the native and unfolded states, respectively, ΔH_U is the free enthalpy of unfolding, Δ_{CPU} is the heat capacity of unfolding, T_m is the transition midpoint temperature, T is temperature, and R represents the universal gas constant.

2.3. Fluorescence spectroscopy

Fluorescence spectra were monitored at 30°C in a JASCO FP-6500 spectrophotometer with an excitation wavelength of 280 nm. The fluorescence emission spectra of samples with a protein concentration of 50 µg/ml were recorded from 300 to 400 nm at a scanning speed of 240 nm/min. The red shift and the change in fluorescence intensity were analyzed together using the average emission wavelength (AEW) (λ) according to Eq. 2 [6].

$$\langle \lambda \rangle = \frac{\sum_{i=\lambda_1}^{\lambda_N} (F_i \cdot \lambda_i)}{\sum_{i=\lambda_1}^{\lambda_N} F_i} \quad (2)$$

in which F_i is the fluorescence intensity at the specific emission wavelength (λ_i).

2.4. GdnHCl-induced denaturation and analysis of unfolding data

The parental and mutant *Bl*ALDHs were unfolded with different concentrations of GdnHCl in 50 mM sodium phosphate buffer (pH 7.0) at room temperature. A 30-min incubation was found to be sufficient for the unfolding process. Unfolding curves of CD and fluorescence spectroscopy were used to calculate the thermodynamic parameters by global fitting of the experimental data. The two-state unfolding model was described by Eq. 3 [7].

$$y_{obs} = \frac{(y_N + m_f[D]) + (y_U + m_u[D]) \cdot \exp[-(\Delta G_{H_2O} - m[D])/RT]}{1 + \exp[-(\Delta G_{H_2O} - m[D])/RT]} \quad (3)$$

where y_{obs} is the observed biophysical signal, y_N and y_U are the intercepts, $\boldsymbol{m}_f$ and $\boldsymbol{m}_u$ are the slopes of the pre- and post-transition baselines, T is temperature, $\boldsymbol{R}$ is the universal gas constant, [D] is the concentration of GdnHCl, ΔG_{H_2O} represents an estimate of the conformational stability of the protein in the absence of denaturant, and $\boldsymbol{m}$ is a measure of the dependence of $\Delta \boldsymbol{G}$.

3. Result

3.1. Enzyme kinetic characterization

Table 1 Specific activity and kinetic parameters of parental and mutant *Bl*ALDHs

Protein	Specific activity (U/mg)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
*Bl*ALDH	8.45 ± 0.03	0.19 ± 0.01	8.33 ± 0.05	43.8
G211A	1.02 ± 0.02	1.13 ± 0.13	1.20 ± 0.07	1.1
G211E	–[a]	ND[b]	ND	ND
G211R	–	ND	ND	ND
G213A	3.11 ± 0.01	1.03 ± 0.15	3.28 ± 0.01	3.2
G213E	–	ND	ND	ND
G213R	–	ND	ND	ND
G217A	3.67 ± 0.02	0.98 ± 0.01	4.16 ± 0.12	4.3
G217E	–	ND	ND	ND
G217R	–	ND	ND	ND
G233A	–	ND	ND	ND
G233E	–	ND	ND	ND
G233R	–	ND	ND	ND
G238A	0.84 ± 0.01	0.07 ± 0.01	0.81 ± 0.02	13.5
G238E	–	ND	ND	ND
G238R	–	ND	ND	ND

[a] –, not detected.

[b] ND, not determined.

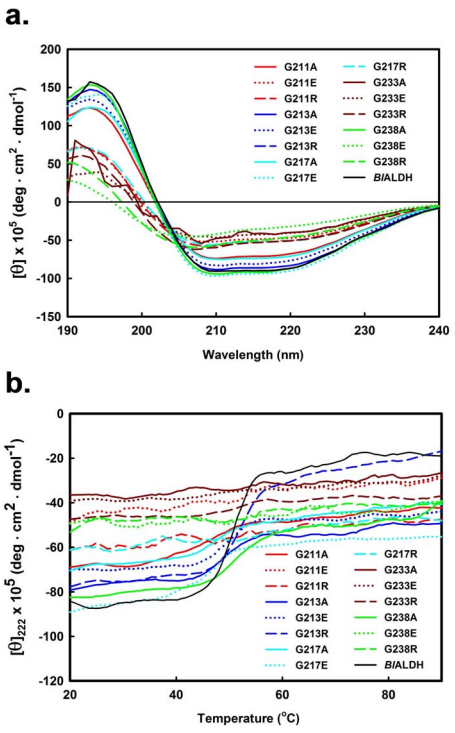

Fig. 2

The assay for dehydrogenase activity revealed that the purified *Bl*ALDH had a specific activity of 8.45 U/mg protein. Replacement of Gly211, Gly213, Gly217, and Gly238 with Ala resulted in a mutant enzyme with more than 56.4% reduction of the ALDH activity, but the remaining mutant enzymes were completely inactive under the assay conditions (Table 1).

3.2. Circular dichroism analysis of the parental and mutant enzymes

To determine if the differences in catalytic activity between the parental and mutant *Bl*ALDHs were due to the secondary structural changes of mutant proteins, we recorded their far-UV CD spectra in the wavelength range of 190–250 nm. In general, the CD spectra of the Ala-substituted variants were similar to that of *Bl*ALDH (Fig. 2a). The parental enzyme has two ellipticity minima of almost equal amplitude at 208 and 218 nm, whereas the negative ellipticity of G211R, G233R, G238E and G238R at 208 nm was more pronounced than at 218 nm (Fig. 2a).

To probe the structural integrity of the parental and mutant enzymes, their thermal denaturation patterns were determined by following the changes in ellipticity at 222 nm (Fig. 2b). The catalytically active mutant enzymes had their T_m values similar to that of the parental protein; however, the midpoint temperature for most of the inactive variants was significantly reduced (Table 2).

Table 2 Thermodynamic parameters of the parental and mutant enzymes

Protein	T_m (°C)[a]	ΔH_{nu} (kcal/mol)[b]	ΔG_{nu} (kcal/mol)[c]
*Bl*ALDH	50.77 ± 0.03	107.48 ± 5.44	5.00
G211A	42.68 ± 0.25	23.71 ± 1.81	0.63
G211E	41.62 ± 0.13	47.30 ± 3.02	1.34
G211R	43.26 ± 0.58	21.95 ± 2.21	0.68
G213A	48.23 ± 0.07	52.31 ± 2.68	1.46
G213E	49.55 ± 0.06	93.08 ± 3.59	3.99
G213R	52.14 ± 2.66	64.59 ± 1.95	3.11
G217A	48.42 ± 0.12	40.43 ± 5.24	1.96
G217E	45.65 ± 0.08	39.37 ± 1.97	2.04
G217R	44.81 ± 2.36	20.65 ± 1.65	0.93
G233A	–	–	–
G233E	–	–	–
G233R	–	–	–
G238A	52.13 ± 0.04	46.47 ± 2.30	3.66
G238E	–	–	–
G238R	–	–	–

[a] T_m, midpoint of the thermal unfolding transition.
[b] ΔH_{nu}, the difference in van't Hoff enthalpy between the native and unfolded proteins.
[c] ΔG_{nu}, the difference in free energy between the native and unfolded proteins.

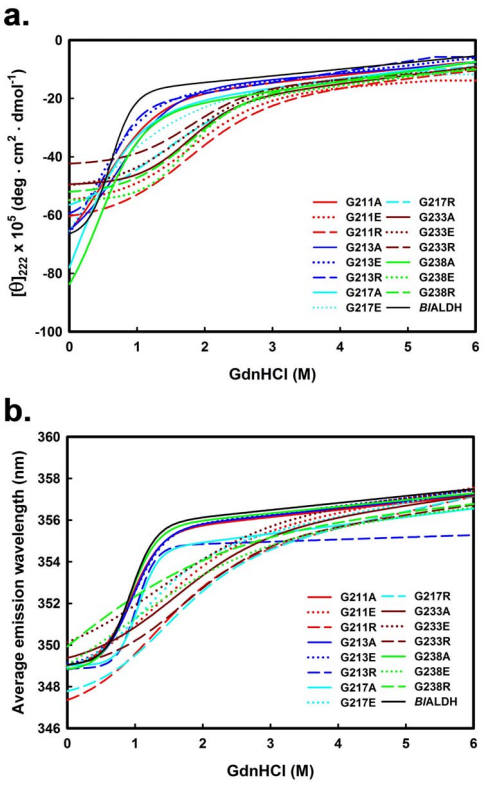

Fig. 3

The thermodynamic parameters of the denaturation process were also analyzed. As shown in Table 4, the ΔH value of *Bl*ALDH was calculated to be 107.5 kcal/mol. However, this value was decreased to <93.1 kcal/mol in the mutant enzymes. Proteins are only marginally stable since the free energy of stabilization ($\Delta G_{N\rightarrow U}$) ranges within 7.1–15.4 kcal/mol and is therefore equivalent to only a few weak bonds [8]. Therefore, it is not surprising that their stability can be weakened through the lack of a few intramolecular interactions. A significant reduction in $\Delta G_{N\rightarrow U}$ values of the variants reflects that these mutant proteins are unstable in the native state.

3.3. Unfolding of the parental and mutant enzymes by GdnHCl

GdnHCl-induced unfolding of *Bl*ALDH and its mutant enzymes was carried out to explore the effect of this denaturant on their secondary structures. The effect of increasing GdnHCl concentrations on the ellipticity of *Bl*ALDH at 222 nm is illustrated in Fig. 3a. By fitting with Eq. 3, *Bl*ALDH showed $[GdnHCl]_{0.5,N\text{-}U}$ of 0.61 M, corresponding to a free energy change of 2.14 kcal/mol for the N → U process. The $[GdnHCl]_{0.5,N\text{-}U}$ value of fifteen mutant enzymes was in the range of 0.29–1.74 M equivalent to a free energy change of 0.45–4.52 kcal/mol.

*Bl*ALDH has a total of five tryptophan residues in its primary sequence. Unfolding of *Bl*ALDH and its variants at different concentrations of GdnHCl was therefore performed and the AEW value that reported on the changes in both fluorescence wavelength and intensity was used to calculate the free energy change ($\Delta G_{N\text{-}U}$) of the unfolding process. As shown in Fig. 3b, native *Bl*ALDH started to unfold at 0.21 M denaturant with $[GdnHCl]_{0.5,N\text{-}U}$ of 0.92 M. By fitting with Eq.3, the $\Delta G_{N\text{-}U}$ value for the N → U process was calculated to be 2.98 kcal/1 mol. Also, the fluorescence signal of GdnHCl-induced variants appeared to be a monophasic process in which their $[GdnHCl]_{0.5,N\text{-}U}$ values were 0.45–1.60 M, corresponding to $\Delta G_{N\text{-}U}$ of 0.31–4.35 kcal/mol, for the N → U process. Based on these observations, it allows us to conclude that the rigidity of *Bl*ALDH structure has been changed by the mutations.

4. Conclusion

In conclusion, potentially important glycine residues for the putative cofactor-binding site of *B. licheniformis* ALDH were identified by bioinformatics methods, which were useful for studying the structure-function relationship of the enzyme in the absence of a three-dimensional structure. Site-directed mutagenesis approach provided the definitive evidence that residues Gly211, Gly213, Gly217, Gly233, and Gly238 were critical for the functionality and stability of *Bl*ALDH. The conservation of these five residues suggested that their roles could be extended to the entire ALDH family. Ongoing experiments in our laboratories endeavor to probe the functional role of residues Val209, Ile229, and Gly242 in *Bl*ALDH. The resultant information will contribute to our greater understanding of the $NAD(P)^+$- binding site of the ALDH family.

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