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論　文

Stabilization Mechanism of Chloride Ion on Thermal Denaturation of *Arthrobacter* Sarcosine Oxidase

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Stabilization mechanism of chloride ion on thermal denaturation of *Arthrobacter* sarcosine oxidase (SoxA) was investigated by calorimetric assays. The thermal unfolding of SoxA observed by differential scanning calorimetry was apparently irreversible. The temperature of unfolding was higher as higher concentration of chloride ions. The temperature of unfolding in the presence of chloride ions at a concentration of 1 M increased by 14 ℃, compared to the case in the absence of chloride ions. Isothermal titration calorimetry showed that a SoxA molecule bound a chloride ion with the dissociation constant of 86 μM. These calorimetric results suggested that a simplest mechanism that can explain the thermal unfolding of SoxA in the presence of chloride ion is as follows: $NCl^- \rightleftarrows U + Cl^-$ and $U \rightarrow U_F$, where N, U, and U_F represent SoxA in a native, reversibly unfolded, and irreversibly unfolded state, respectively. In this model, SoxA undergoes reversible unfolding with simultaneous dissociation of a chloride ion and subsequently the irreversible process occurs. The addition of a chloride ion shifts the equilibrium to favor the native-state side, resulting in an apparent stabilization.

Keywords: calorimetry; chloride ion; protein stability; sarcosine oxidase; thermal denaturation

1. INTRODUCTION

Sarcosine oxidase [EC 1.5.3.1] catalyzes the oxidative demethylation of sarcosine to produce glycine, formaldehyde, and hydrogen peroxide.[1] This enzyme is widely used as a diagnostic reagent for determining creatinine and creatine, which are reference markers of kidney dialysis.[2] Sarcosine oxidase from *Arthrobacter* sp. TE1826 (SoxA) is a 388 amino acid polypeptide that is covalently bound to FAD (flavin adenine dinucleotide).[3] SoxA is stable in the pH range 6.5-10 and has activity optimum at pH 7.5 ~ 8.0.[4] Although SoxA is sensitive to heat, a chloride ion (KCl) can remarkably improve thermal stability of the enzyme.[4] For example, SoxA activity is destroyed by incubation at 60 ℃ for 10 min in the absence of chloride ion, but 80 % activity remains when the incubation is carried out in the presence of 10 mM chloride ion.[5] Although the addition of salt is useful for stabilizing the enzyme, how stabilization is brought about at a mechanistic level has not been elucidated. In this study, the mechanism of stabilization of SoxA by chloride ions was investigated via differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC).

2. MATERIALS and METHODS

2.1 Sarcosine oxidase sample

A lyophilized preparation of wild type *Arthrobacter* SoxA and the mutant form Cys265Ser were prepared as reported previously.[3] The concentration of SoxA was determined spectrophotometrically using the molar absorption coefficient $\varepsilon = 69{,}200\ M^{-1}\ cm^{-1}$ at 280 nm, which was calculated on the basis of the amino acid composition and absorbance of FAD.[6] Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Bruker Daltonics

autoflex LRF (Billerica, MA, USA). The molecular weight of SoxA was determined to be 43,850, which is consistent with the calculated molecular weight based on the amino acid composition of SoxA plus FAD (43,880).

Potassium chloride (KCl; Wako Co., Osaka, Japan) was used as a source of chloride ions. Unless otherwise noted, experiments were done in a sodium phosphate buffer, pH 8.0, at a concentration of 20 mM.

2.2 Thermal inactivation

The level of thermal inactivation was determined by measuring the activity of the enzyme remaining after treatment, as follows. First, the enzyme solution at 4 ℃ was heated up to desired temperatures at a rate of 1 K min^{-1} and an appropriate amount of the enzyme solution was recovered at the temperatures. Next, the heat-treated enzyme solutions were immediately cooled on ice water and enzymatic activity was measured using the 4-aminoantipyrine and peroxidase system method.[3)]

2.3 Differential scanning calorimetry

Excess specific heat capacity due to thermal unfolding of SoxA was observed using a differential scanning calorimeter (model VP-DSC from MicroCal LLC, Northampton, MA, USA). Unless otherwise noted, the data were obtained at a scan rate of 1 K min^{-1}. The protein concentration was 4 μM. The protein sample was dialyzed against the buffer solution twice and the final dialysate was used as a reference solution for DSC scans. The observed DSC data were analyzed using Origin 5.0 software (MicroCal) with an add-in program.[7-10)]

2.4 Isothermal titration calorimetry

Isothermal binding of SoxA to a chloride ion was determined using an isothermal titration calorimeter (VP-ITC, MicroCal) at 45 ℃. ITC data were analyzed using Origin 5.0 with an add-in program (MicroCal). A 5 mM sodium phosphate buffer, pH 8.0, was used. Either 5 μl (initial 3 injections) or 10 μl (following 22 injections) of potassium chloride solution (4 mM) were titrated to the SoxA solution (25 μM).

2.5 Circular dichroism spectroscopy

Far-UV circular dichroism (CD) spectra of SoxA in the absence and presence of KCl (1 M) at 20 ℃ were obtained using a CD spectropolarimeter (JASCO J-820, JASCO Co., Tokyo, Japan). Spectra were recorded between 200～250 nm using a quartz cell with a 1 mm light path length and a scan speed of 50 nm min^{-1}. Ten spectra were accumulated and averaged for each sample. The protein concentration was 1.6～3.1 μM.

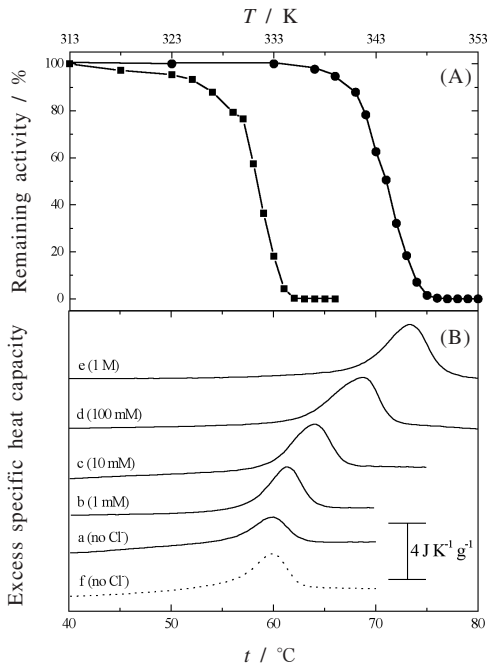

Fig.1 Influence of chloride ions on thermostability of SoxA. (A) Thermal inactivation of SoxA in the absence (solid squares) or presence (solid circles) of 1 M KCl. The remaining activity of the protein in solution, which was heated up at a rate of 1 K min^{-1}, was measured. (B) DSC curves for unfolding in the absence or presence of chloride ions (Cl^-). Solid lines, DSC traces for wild type SoxA. The Cl^- concentrations were as follows: 0 (a), 1 (b), 10 (c), 100 mM (d), and 1 M (e). Dotted line (f), a DSC trace for a mutant form of SoxA, Cys265Ser. Observed at pH 8.0. The protein concentration was 4 μM in each case.

3. RESULTS and DISCUSSION

3.1 Thermal inactivation

The level of thermal inactivation of SoxA enzyme was determined after heating the enzyme solution at a rate of 1 K min^{-1}. As shown in **Fig.1(A)**, in the absence of KCl, the inactivation temperature t_d, at which the remaining activity becomes 50 %, was 58 ℃, whereas

Table 1 Thermodynamic parameters of thermal unfolding of SoxA in the absence and presence of chloride ions at pH 8.0. [a)] Values are given with the standard errors from the fit.

$[Cl^-]$ / mM	$t_{1/2}$ [a)] / ℃	Δh_{cal} [a)] / J g^{-1}	ΔH_{cal} [a)] / kJ mol^{-1}	$\Delta H_{vH}/\Delta H_{cal}$	Δc_p at $t_{1/2}$ / J K^{-1} g^{-1}	ΔC_p at $t_{1/2}$ / kJ K^{-1} mol^{-1}
0	58.9 ± 0.1	10.5 ± 0.2	459 ± 11	1.5	0.19	8.5
1	60.9 ± 0.1	13.4 ± 0.4	587 ± 16	1.4	0.42	18
10	63.6 ± 0.0	14.2 ± 0.3	624 ± 14	1.3	0.28	12
100	67.9 ± 0.1	19.5 ± 0.6	855 ± 26	0.74	0.31	14
1000	72.8 ± 0.1	17.8 ± 0.5	781 ± 23	0.91	0.80	35

the value of t_d increased to 71 ℃ in the presence of 1 M KCl.

3.2 Thermal unfolding observed by DSC

The dependence of excess specific heat capacity due to thermal unfolding of SoxA on temperature, as observed by DSC in the absence and presence of various concentrations of chloride ions (KCl), is shown in **Fig.1(B)(a-e)**. A single endothermic peak was observed in each case. The sample solutions were cooled after the completion of each scan in the calorimeter cell and immediately rescanned, but no endothermic peaks were observed, implying that the thermal unfolding was apparently irreversible. In the absence of KCl, no significant dependence of the peak temperature t_p on the concentration of the enzyme (4 ~ 94 μM) was observed. However, the value of t_p increased with increasing concentrations of KCl. When sodium chloride (NaCl) was used as a source of chloride ions in DSC experiments, t_p was also higher with higher concentration of NaCl. DSC scans were also carried out using a potassium phosphate buffer, pH 8.0, at concentrations of 20 and 100 mM. DSC curves obtained were identical to that observed in a sodium phosphate buffer containing no chloride ion, that is, varying the concentration of potassium ion had no effect on the value of t_p. These results indicate that the stabilization of SoxA by KCl is attributed to chloride ion and not to potassium ion.

The specific enthalpy of unfolding in J g^{-1} units, Δh_{cal}, was determined from the area of the DSC traces using a curve-fitting method.[7-10)] In the absence of KCl, Δh_{cal} was about 10 J g^{-1} (**Table 1**), which is a relatively small value for a globular protein.[11)] Values of $t_{1/2}$, at which unfolding is half completed under the conditions of the assay, and Δc_p, the change in heat capacity between the native and unfolded states at $t_{1/2}$, were determined (**Table 1**). The table also lists the values of ΔH_{cal}, the molar calorimetric enthalpy (= Δh_{cal} × molar mass), and ΔC_p, the molar heat capacity change. In the presence of 5 mM FAD, no change in $t_{1/2}$ or Δh_{cal} was observed, which indicates that FAD does not dissociate upon thermal denaturation of SoxA. Effects of other halogen ions were examined and halogen ions, such as F^-, Br^-, and I^-, were also able to stabilize the protein. The value of t_p in the presence of 100 mM of each ion was 62.2 (F^-), 68.1 (Br^-,) and 61.2 ℃ (I^-) (data not shown).

No siginificant difference was observed between the values of t_d obtained in the inactivation experiments (*i.e.* about 58 ℃ in the absence of Cl^- and 71 ℃ in the presence of 1 M Cl^-) and the $t_{1/2}$ values obtained by DSC (*i.e.* about 59 and 73 ℃). It is likely that the loss of activity and the global collapse of SoxA are concomitant phenomena.

3.3 Binding equilibrium of chloride ion

The binding parameters for association of SoxA with chloride ion were evaluated using ITC. The binding isotherm at pH 8.0 and 45 ℃ is shown in **Fig.2**. The average number of chloride ions that bind to the SoxA molecule was determined to be 0.87 ± 0.25, which is close to unity, that is, one chloride ion binds to a SoxA molecule. When the ITC data was analyzed by assuming 2:1 or 3:1 binding of chloride ions to a SoxA molecule, no reasonable fit was obtained between the observed data and the theoretical values. Structural analysis shows that sarcosine oxidase from *Bacillus* sp. B-0618 contains one bound chloride ion.[12,13)] Similar situations may be expected for the case of the binding of SoxA and chloride ion. The dissociation constant K_d for SoxA-chloride ion was 86 μM. This K_d value implies that the increase of t_p (or $t_{1/2}$) observed with increased concentrations of KCl (**Fig.1(B)**) is not due to structural changes in SoxA, if

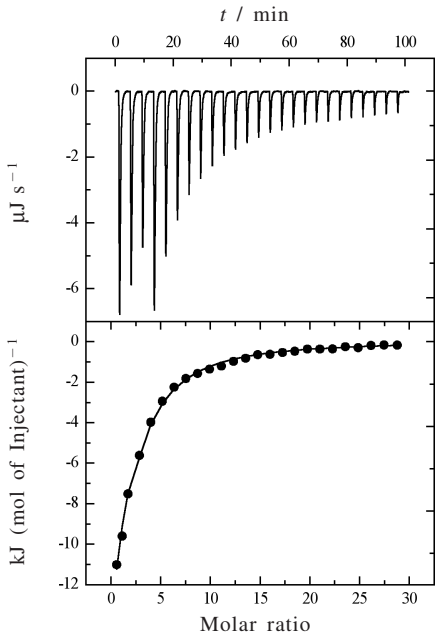

Fig.2 Isothermal titration calorimetry of the binding of chloride ions to SoxA. Upper panel, thermogram; lower panel, binding isotherm. In the binding isotherm, solid circles show experimental results and solid line shows a theoretical curve determined using a one-site binding model. Observed at pH 8.0 and 45℃. Either 5 μl (initial 3 injections) or 10 μl (following 22 injections) of a 4 mM potassium chloride solution were added to the 25 μM SoxA solution.

any, as most (92 %) SoxA molecules formed a complex with a chloride ion even at the lowest concentration tested (1 mM KCl), and as the value of t_p increases with the increasing concentrations of KCl.

The binding enthalpy change ΔH was evaluated from the ITC trace data to be -62.6 kJ mol^{-1}, and the standard Gibbs energy change of binding ΔG° was calculated to be -24.7 kJ mol^{-1} from $\Delta G^\circ = RT \ln K_d$, where R is the gas constant and T the absolute temperature. Additionally, the value of $T\Delta S^\circ$, where ΔS° is the standard entropy change of binding, was calculated to be -37.9 kJ mol^{-1} from $T\Delta S^\circ = \Delta H - \Delta G^\circ$. The binding of chloride ion is thus an enthalpy-driven reaction.

3.4 The mechanism of SoxA stabilization by chloride ion

Thermal unfolding of SoxA was apparently irreversible, and the peak temperature of the DSC traces increased with increasing concentrations of KCl. Continuing changes in t_p were observed even at concentrations of chloride ions at which the proteins were nearly fully saturated with chloride ions. Similar phenomena were observed with apparently irreversible unfolding studied by DSC.[9,14-16] Furthermore, in the absence and presence of chloride ions, no significant dependence of the peak temperature on the scan rate (0.5 ~ 1.5 K min^{-1}) was observed. These observations would not have been obtained if the reaction was simply irreversible; for example, $NCl^- \rightarrow D + Cl^-$, where N and D represent SoxA in the native and unfolded state, respectively.

A simplest mechanism that can explain the thermal unfolding of SoxA in the presence of chloride ions can thus be considered to consist of two steps, as follows: $NCl^- \rightleftarrows U + Cl^-$ and $U \rightarrow U_F$, where U, and U_F are the reversibly and irreversibly unfolded states of SoxA, respectively. In this model, there is a dissociation equilibrium between the protein and Cl^- and subsequently the irreversible process occurs. A chloride ion stabilizes a SoxA molecule by shifting the equilibrium to favor the native-state side. This model does not necessitate the structural stabilization of the SoxA molecule by the binding of a chloride ion, which is consistent with the finding that no difference was detected between the far-UV CD spectra in the absence versus in the presence of 1 M KCl (data not shown).

3.5 Possibility of dimerization

If we assume that the DSC curves can be analyzed using the van't Hoff equation, and that the second irreversible step does not affect the analysis significantly as was mentioned earlier,[8] we can determine the ratio of the two enthalpy changes, $\Delta H_{vH}/\Delta H_{cal}$, where ΔH_{vH} is the van't Hoff enthalpy. The values of the ratio are listed in **Table 1**. In the absence of KCl, the ratio is 1.5, which is significantly greater than unity. If the assumptions stated above are valid, this suggests that there are positive interactions among SoxA molecules, such as dimerization.[8] In DSC measurements, however, the dependence of the peak temperature on the protein concentration was not observed, suggesting that a dimer via non-covalent interactions is not formed. On the other hand, SoxA has a free cysteine residue, Cys265, on the

protein surface[17] and it seems possible that a dimer is formed via an intermolecular disulfide bridge. To exclude the possibility of the formation of the intermolecular disufide bridge, we observed thermal unfolding of a mutant protein, Cys265Ser (**Fig.1(B)**, **f**). The DSC trace obtained was essentially the same as that of the wild type enzyme and the ratio $\Delta H_{vH}/\Delta H_{cal}$ was 1.6. This implies that the deviation of the value of $\Delta H_{vH}/\Delta H_{cal}$ from unity is not due to the formation of the intermolecular disulfide bridge but instead, implies at least one of the assumptions used for analysis of the DSC data for SoxA is not a valid assumption.

In conclusion, the calorimetric studies here suggested that the thermal unfolding of SoxA in the presence of chloride ions involves a dissociation equilibrium between SoxA and Cl^-, followed by the irreversible step. The stabilization of SoxA by chloride ion is due to a reversal of the dissociation equilibrium to favor the native-state side.

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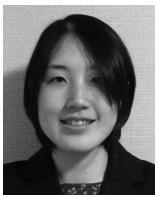

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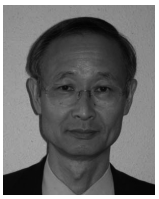

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