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Calcium-binding and structural stability of echidna and canine milk lysozymes

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Abstract

For echidna and canine milk lysozymes, which were presumed to be the calcium-binding lysozymes by their amino acid sequences, we have quantitated their calcium-binding strength and examined their guanidine unfolding profiles.

The calcium-binding constants of echidna and canine lysozymes were determined to be $8.6 \times 10^6 \text{ M}^{-1}$ and $8.9 \times 10^6 \text{ M}^{-1}$ in 0.1 M KCl at pH 7.1 and 20 °C, respectively. The unfolding of decalcified canine lysozyme proceeds in the same manner as that of α -lactalbumin, through a stable molten globule intermediate. However, neither calcium-bound nor decalcified echidna lysozyme shows a stable molten globule intermediate. This unfolding profile of echidna lysozyme is identical to that of conventional lysozymes and pigeon egg-white lysozyme, avian calcium-binding lysozyme. This result supports the suggestion of Prager and Jolles (Prager EM, Jolles P. 1996. Animal lysozymes c and g: An overview. In: Jolles P, ed. *Lysozymes: Model enzymes in biochemistry and biology*. Basel-Boston-Berlin: Birkhauser Verlag. pp 9–31) that the lineage of avian and echidna calcium-binding lysozymes and that of eutherian calcium-binding lysozymes diverged separately from that of conventional lysozymes.

Keywords: calcium-binding; canine; equine; lysozyme; molecular evolution; molten globule

Chicken-type (c-type) lysozymes and α -lactalbumins are believed to have evolved from a common ancestral protein because of the similarity of their amino acid sequences (Brew et al., 1970), conformation (Stuart et al., 1986; Acharya et al., 1989; Pike et al., 1996), and intron-exon constitution of their genes (Qasba & Safaya, 1984). However, the biological functions of these proteins are quite different. Lysozyme is a lytic enzyme, which catalyzes the hydrolysis of the $\beta(1-4)$ glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in the main polysaccharide constituent of Gram-positive bacterial cell walls. In contrast, α -lactalbumins regulate lactose biosynthesis by modulating the specificity of β -galactosyltransferase.

Another property that distinguishes these two proteins qualitatively is their calcium-binding ability: c-type lysozyme does not specifically bind calcium ions, while α -lactalbumin binds it tightly (Hiraoka et al., 1980). The three-dimensional structure of α -lactalbumin was determined by X-ray crystallographic study (Stuart et al., 1986; Acharya et al., 1989; Pike et al., 1996). These results revealed that the calcium-binding site is composed of three carboxylate groups of aspartyl residues (82, 87, and 88 in α -lactalbumin), carbonyl oxygen atoms from residues 79, 84, and

two water molecules. Comparative amino acid sequence analysis of lysozymes and α -lactalbumins has revealed that the corresponding aspartyl residues 82, 87, and 88 are conserved in equine milk and pigeon egg white lysozymes (Table 1). Consequently, it was suggested that these lysozymes might bind a calcium ion (Stuart et al., 1986), which was confirmed by Nitta et al. (1987, 1988).

Besides the difference as described above, another notable difference between c-type non-calcium-binding (conventional) lysozymes and α -lactalbumins is in their unfolding profiles. A stable equilibrium intermediate, molten globule state is observed during the unfolding of α -lactalbumin by guanidine hydrochloride (GdnHCl) (Kuwajima et al., 1976), but conventional lysozymes generally unfold according to a cooperative two-state model and only an unstable intermediate is transiently observable by kinetic methods (Kuwajima et al., 1985). The unfolding profiles of calcium-binding lysozymes, equine milk, and pigeon egg-white lysozymes have been investigated recently, and only the mammalian, equine milk lysozyme unfolds through a stable molten globule intermediate (Nitta et al., 1993; Van Dael et al., 1993).

It is also suggested that there are other mammalian calcium-binding lysozymes, e.g., from echidna and canine milk (Teahan et al., 1991; Grobler et al., 1994). Echidna is a member of monotreme, egg-laying mammals. The relationships between monotreme proteins and those of other mammals are attractive for the study of molecular evolution (Teahan et al., 1991).

The aim of this investigation is to quantitate the calcium-binding strength and to examine the unfolding profiles of these lysozymes.

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Abbreviations: GdnCl, guanidinium chloride; EML, echidna milk lysozyme; CML, canine milk lysozyme; CD, circular dichroism.

Table 1. Amino acid sequences of the calcium-binding sites^a

	81			85				90		91	93		
Echidna lysozyme	Ser	Lys	Leu	Leu	Asp	Asp	Asp	Ile	Thr	Asp	Asp	Leu	Lys
Canine lysozyme	Ser	Lys	Phe	Leu	Asp	Asp	Asn	Ile	Asp	Asp	Asp	Ile	Ala
Equine lysozyme	Ser	Lys	Leu	Leu	Asp	Glu	Asn	Ile	Asp	Asp	Asp	Ile	Ser
Pigeon lysozyme	Ser	Lys	Leu	Arg	Asp	Asp	Asn	Ile	Ala	Asp	Asp	Ile	Gln
Bovine α -lactalbumin	Asp	Lys	Phe	Leu	Asp	Asp	Asp	Leu	Thr	Asp	Asp	Ile	Met
Human α -lactalbumin	Asp	Lys	Phe	Leu	Asp	Asp	Asp	Ile	Thr	Asp	Asp	Ile	Met
Chicken lysozyme	Ser	Ala	Leu	Leu	Ser	Ser	Asp	Ile	Thr	Ala	Ser	Val	Asn
Human lysozyme	Ser	Ala	Leu	Leu	Gln	Asp	Asn	Ile	Ala	Asp	Ala	Val	Ala

^aThe residue numbering is based on that for echidna and canine milk lysozymes.

^bThe calcium ligands are Asp carboxylate groups.

^cThe calcium ligands are main-chain carbonyl groups.

Although studies of the calcium-binding of these proteins with site-directed mutagenesis have recently been published (Haezebrouck et al., 1993; Anderson et al., 1997), studies of naturally occurring proteins are also important to understand the significance and function of such properties.

Results

Calcium-binding property

The binding of calcium ions to EML and CML was examined by competition titrations with the calcium-binding dye, Quin-2 in 0.01 M HEPES, 0.1 M KCl at pH 7.1 and 20 °C (Nitta et al., 1988). The number of the binding sites n and binding constant of calcium ions to lysozymes K_{Ca} were determined by the following equation obtained previously (Nitta et al., 1988):

$$[Ca]_f = (1 - F)/(FK_d) + (1 - F)[Quin2]_f + (nK_{Ca}(1 - F)[L]_f)/(FK_d + (1 - F)K_{Ca}) \quad (1)$$

where F is the free fraction of Quin-2, which can be calculated from the change of the absorbance at 354 nm, $[Ca]_f$, $[Quin2]_f$, and $[L]_f$ are total concentrations of calcium ions, Quin-2, and lysozyme, respectively. The results are shown in Figure 1. Solid lines represent the best-fitted curves calculated by a trial-and-error method. The calculated n and K_{Ca} values are given in Table 2. For the purpose of comparison, the n and K_{Ca} values of equine lysozyme, pigeon lysozyme, and bovine α -lactalbumin, which were determined previously in the same manner as shown above, are also included in Table 2.

Circular dichroism

Figures 2 and 3 show the far (210–250 nm) and near (270–320 nm) UV circular dichroism (CD) spectra of EML and CML about apo-form and holo-form in native conditions (0.1 M TES buffer, pH 7.2, 25 °C), respectively. Solid lines represent the spectra of apolysozymes with 1 mM EDTA. Dashed lines are the spectra of hololysozymes with 1 mM EDTA and 2 mM $CaCl_2$. The solutions of hololysozyme were prepared by adding concentrated solution of $CaCl_2$ to the solution of apolysozyme with EDTA. For both lyso-

zymes, essentially the same spectra as that of native hololysozyme was obtained in the absence of calcium ion.

GdnHCl induced unfolding

The unfolding of these lysozymes was monitored by recording the CD ellipticity at 222 and 294 nm. These spectral parameters are commonly used as a measure of the extent of secondary and tertiary structures, respectively.

The apparent fractional extent of unfolding f_{app} was calculated from the ellipticity values using the equation: $f_{app} = ([\theta]_N - [\theta]) / ([\theta]_N - [\theta]_U)$, where $[\theta]$ represents the observed ellipticity under given conditions, and $[\theta]_N$ and $[\theta]_U$ are ellipticity values in the native and the unfolded states, respectively.

Figure 4 shows the normalized transition curves of the unfolding of the echidna apo-(A) and hololysozyme (B), which are expressed

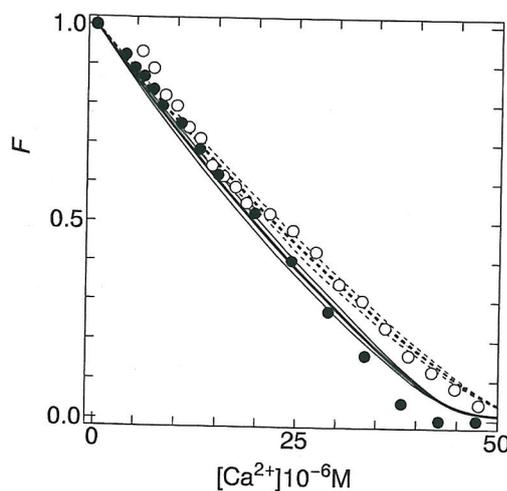


Fig. 1. Titration of Quin-2 in the presence of EML (open circles) and CML (filled circles). Free fraction (F) of Quin-2 is plotted against the total concentration of calcium ions. The concentrations of Quin-2 were 15.5 μ M for EML and 13.5 μ M for CML, respectively. The concentrations of lysozymes were 34.5 and 26.6 μ M for EML and CML, respectively. Thick lines are the best fitted curves calculated with the parameters listed in Table 1 by Equation 1. Accompanying thin lines are calculated using the binding constants 1×10^6 M less/more than the best fitted values.

Table 2. Binding constants of a calcium ion to lysozymes and α -lactalbumin

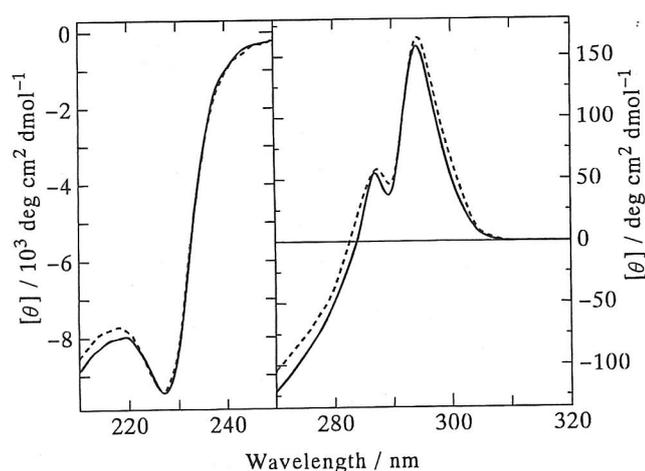
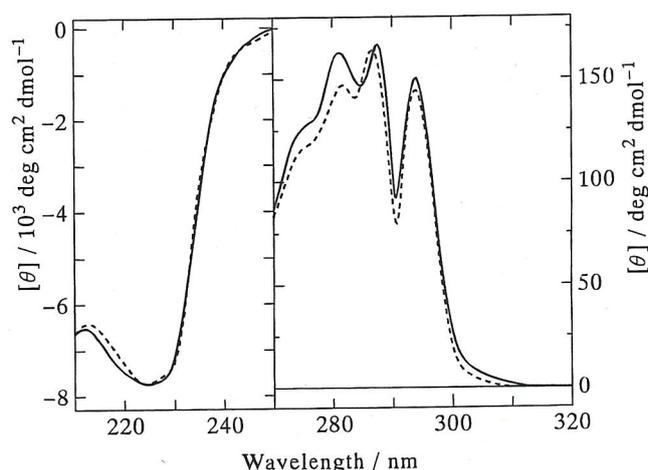
	n	K_{Ca}/M^{-1}
Canine lysozyme	1.16	8.9×10^6
Echidna lysozyme	1.02	8.6×10^6
Equine lysozyme ^a	1.00	2.0×10^6
Pigeon lysozyme ^a	0.79	1.6×10^7
Bovine α -lactalbumin ^a	0.83	4.0×10^7

^aData from Nitta et al. (1988).

in terms of f_{app} vs. GdnHCl concentration. The unfolding profile of EML showing coincident transition curves derived from the ellipticity changes at 222 and 294 nm is well represented as a two-state process in which tertiary and secondary structure are lost simultaneously and cooperatively. Values of f_{app} both at 294 and 222 nm change at about 1.2 M GdnHCl in apo-form and at about 2.2 M GdnHCl in holo-form.

On the other hand, the transition curves of GdnHCl induced unfolding of CML are different from those of the conventional lysozymes and EML (Fig. 5). In the case of apo CML (Fig. 5), the unfolding proceeds by a two-step process. The values of f_{app} at 294 nm (open circles in Fig. 5) change around 2 M of GdnHCl but that at 222 nm (filled circles in Fig. 5) remain constant. Latter values change around 5 M of GdnHCl. This result suggests that native structure of decalcified CML changes to molten globule state around 2 M GdnHCl and at 5 M GdnHCl the molten globule structure changes to fully unfolded structure. The GdnHCl induced unfolding profiles of holo CML (pH 7.2, 25°C, 1 mM excess $CaCl_2$) are shown in Figure 5. In this case, the stable molten globule state is not observed and unfolding proceeds by a one-step process. Both tertiary and secondary structure change at about 5 M of GdnHCl.

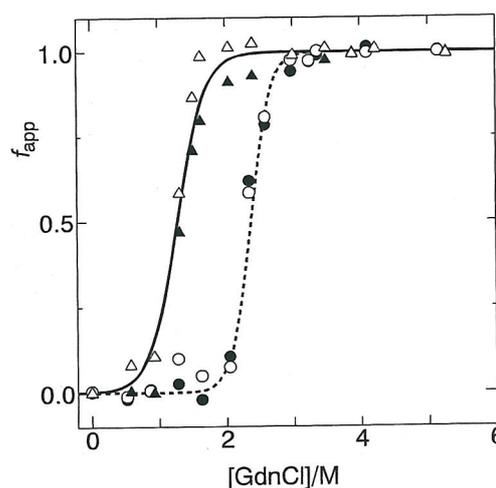
Free energy changes of unfolding, ΔG_u , of canine apolysozymes were estimated from Figure 5, and plotted against the concentra-

**Fig. 2.** Circular dichroism spectra of echidna milk lysozyme. Solid line: apolysozyme in 0.1 M TES (pH 7.2), 0.1 M KCl, 1 mM EDTA. Dashed line: holo-lysozyme in 0.1 M TES (pH 7.2), 0.1 M KCl, 1 mM EDTA, 2 mM $CaCl_2$.**Fig. 3.** Circular dichroism spectra of canine milk lysozyme. Solid line: apolysozyme in 0.1 M TES (pH 7.2), 0.1 M KCl, 1 mM EDTA. Dashed line: holo-lysozyme in 0.1 M TES (pH 7.2), 0.1 M KCl, 1 mM EDTA, 2 mM $CaCl_2$.

tion of GdnCl and shown in Figure 6, respectively. These data were fitted to the following equation with linear least-squares method:

$$\Delta G_u = \Delta G_u^0 - mC, \quad (2)$$

where ΔG_u^0 is the extrapolated free energy change to 0 M GdnCl, m the cooperativity parameter of the unfolding, and C the concentration of GdnCl. The obtained parameters are listed in Table 3 with those of the other calcium-binding lysozymes and α -lactalbumins. In the case of canine lysozyme, native to molten globule transition (open triangles in Fig. 5) and molten globule to unfolded state transition (filled triangles and circles in Fig. 5) were so separated, apparent degree of unfolding f_{app} was used as degree of

**Fig. 4.** GdnCl induced transition curves of echidna milk lysozyme in 0.1 M TES buffer (pH 7.2, 25°C). Triangles: apolysozyme with 1 mM EDTA. Circles: holo-lysozyme with 1 mM EDTA and 2 mM $CaCl_2$. Open and filled symbols illustrate the values of f_{app} calculated from the ellipticity at 294 and 222 nm, respectively. Lines were calculated with the parameters obtained with Equation 2.

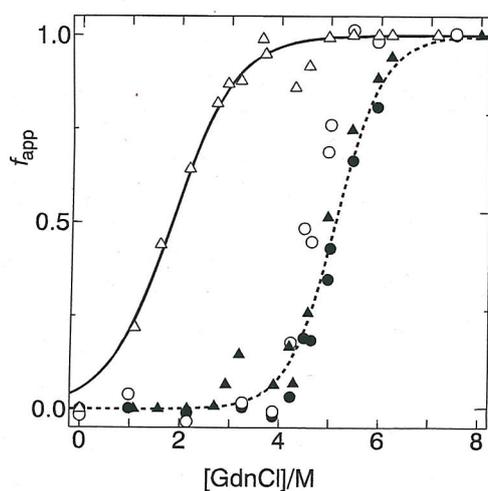


Fig. 5. GdnCl induced transition curves of canine milk lysozyme in 0.1 M TES buffer (pH 7.2, 25 °C). Triangles: apolysozyme with 1 mM EDTA. Circles: holo-lysozyme with 1 mM EDTA and 2 mM CaCl₂. Open and filled symbols illustrate the values of f_{app} calculated from the ellipticity at 294 and 222 nm, respectively. Lines were calculated with the parameters obtained from Figure 6 and shown in Table 3.

unfolding f from native to molten globule, and from molten globule to fully unfolded state, respectively. Because of the shortening of the sample, echidna lysozyme could not be analyzed in that way.

Discussion

Because the amino acid residues that constitute the calcium-binding site of α -lactalbumins and calcium-binding lysozymes are conserved in EML and CML (Table 1), it is suggested that these lysozymes have a similar calcium-binding site as α -lactalbumin and bind a calcium ion. In fact, the structure of calcium-binding site of EML were confirmed by X-ray crystallographic study (Guss et al., 1997). In this study, it is confirmed that EML and CML bind

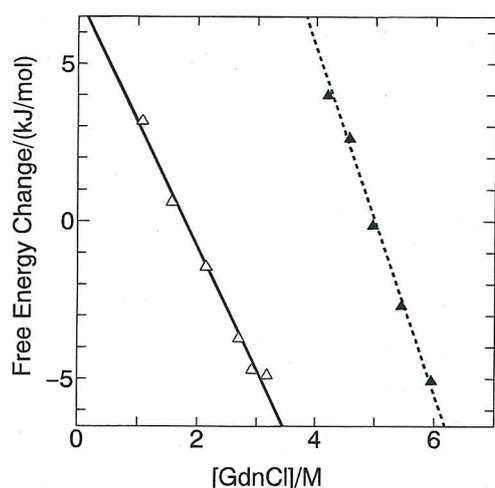


Fig. 6. GdnCl concentration dependence of the free energy changes of the unfolding of canine apolysozyme. Symbols are the same as in Figure 5.

Table 3. Unfolding parameters of calcium-binding lysozymes and α -lactalbumins

	Type of transition ^a	ΔG_0 (kJ/mol)	m (kJ/mol/M)
Canine lysozyme	apo in 1 mM EDTA	7.1 (0.5) ^b	3.94 (0.08)
	A-U	28 (4)	5.52 (0.16)
Pigeon lysozyme ^c	apo in 1 mM EDTA	12.08 (0.06)	6.63 (0.05)
	holo in 0.18 mM CaCl ₂	30.9 (0.7)	9.73 (0.07)
	holo in 10 mM CaCl ₂	36.2 (0.5)	9.73 (0.04)
Equine lysozyme ^d	apo or holo	14.7 (0.3)	3.55 (0.02)
Bovine α -lactalbumin ^e	N-A	14.1-15.1	4.0-6.1
	A-U	8.6	3.7
Human α -lactalbumin ^f	N-A	9.5	8.3
	A-U	13.1	4.0

^aN, A, and U mean native, intermediate, and unfolded states, respectively.

^bNumbers in parentheses are standard deviations.

^cEstimated from Figure 8 of Nitta et al. (1993).

^dEstimated from Figure 1b of Nitta et al. (1993).

^eEstimated for Kita et al. (1976) and Kuwajima et al. (1976).

^fEstimated from Table 1 of Nozaka et al. (1978).

one calcium ion with high affinity at neutral pH as various α -lactalbumins and other calcium-binding lysozymes (Table 2).

Equine milk and pigeon egg-white lysozymes were the widely studied calcium-binding lysozymes (Nitta et al., 1987, 1988, 1993; Desmet et al., 1989). Circular dichroic spectra of these lysozymes were almost the same for the holo and apo state at neutral pH (Desmet et al., 1989; Nitta et al., 1993). In contrast, for α -lactalbumin, the drastic structural change was brought by removal of the bound Ca²⁺ at neutral pH and low salt concentration (Hiraoka & Sugai, 1985). Circular dichroic spectra of EML and CML (Figs. 2, 3) scarcely changed upon decalcification, as well as equine and pigeon lysozymes. It is revealed that native conformation of these lysozymes does not unfold when a bound calcium ion is removed. However, the NMR study of equine lysozyme indicated that a global conformational change arose from the removal of the calcium ion, although the conformational change was not the complete unfolding (Tsuge et al., 1991). In the cases of EML and CML, the same conformational change between the holo-form and apo-form might occur.

In the recent investigation (Nitta et al., 1993), equine lysozyme exhibits a stable molten globule state during unfolding by GdnHCl. This property of the structural transition is the same as that of α -lactalbumin, although there are some differences between the molten globule state of equine lysozyme and that of α -lactalbumin. On the other hand, pigeon lysozyme, calcium-binding avian lysozyme, unfolds by a cooperative two-state mechanism, as conventional lysozyme. Consequently, it is suggested that the avian and mammalian calcium-binding lysozymes can be classified separately by the stability of the folding intermediate. In spite of the absence or presence of Ca²⁺, EML unfolds by a cooperative two-state process (Fig. 4). The GdnHCl concentration of the transition midpoint increases with an increase of the concentration of cal-

cium. It suggests that the calcium binding results in the increase of the structural stability and the calcium-binding site is destroyed by the unfolding. These properties of the unfolding process coincide with that of pigeon lysozyme. In the case of CML, a stable molten globule intermediate is observed during GdnHCl induced unfolding for apolysozyme (Fig. 5). The first process for the unfolding of CML, the transconformation from native to molten globule state, is dependent on the concentration of calcium. At 1 mM CaCl₂, the transition concentration increases to about 5 M GdnHCl, as shown in Figure 5. On the other hand, the second process, from molten globule to unfolded structure, is not dependent on the concentration of calcium. It means that the binding site of a calcium ion is destroyed in the process from native to molten globule state. These phenomena totally coincide with those of equine lysozyme (Nitta et al., 1993).

Although echidna is classified as a mammal, the unfolding profile is similar to that of avian calcium-binding lysozyme rather than that of other mammalian lysozymes. These relations are interesting because of the problem how α -lactalbumin evolved from lysozyme (Nitta & Sugai, 1989). Grobler et al. (1994) and Prager and Jolles (1996) suggested the echidna and placental calcium-binding lysozymes represent distinct lineages and the lineage of echidna and avian calcium-binding lysozymes have separated before the separation of the lineages of birds and mammals. The present result that echidna lysozyme does not show stable molten globule intermediate, like pigeon lysozyme, supports their suggestion.

The stability of the molten globule is undoubtedly determined with its amino acid sequence as that of native conformation. The authors have compared helix propensities and hydrophobicities of conventional lysozymes, calcium-binding lysozymes, and α -lactalbumins that have known amino acid sequences. However, they could not find any differences between them at the present time. We need extensive efforts to collect the experimental helix propensities of the peptide fragments of these proteins.

Equine lysozyme, another eutherian calcium-binding lysozyme, is so unique that some tertiary structure is observed in molten globule state (Nitta et al., 1993; Van Dael et al., 1993; Morozova-Roche et al., 1997). The molten globule state (A-state) is so stable that the midpoint of guanidine unfolding is around 4 M, in which values of bovine and human α -lactalbumin are about 2.5 and 3 M, respectively. In the case of canine lysozyme, the thermodynamic parameters listed in Table 3 indicate that it is also the case for canine lysozyme. The cooperativity parameter m of N-A transition is smaller than that of A-U transition, inverse for the case of bovine and human α -lactalbumins, although the sum of m 's for two transitions roughly coincide with that of N-U transition of pigeon lysozyme. This means that the intermediate, molten globule state of canine lysozyme is more ordered and the hydrophobic side chains are more buried than that of bovine or human α -lactalbumin. The midpoint of A-U transition is as high as 5 M in guanidine unfolding, as shown in Figure 5. It is probably a common characteristics of the eutherian calcium-binding lysozyme that the molten globule state is more stable and more ordered than that of α -lactalbumin.

Materials and methods

There are two variants of echidna milk lysozyme (EML), designated lysozyme I and II, which is isolated from mature milk of the subspecies *Tachyglossus aculeatus multiaculeatus* and *Tachyglossus aculeatus aculeatus*, respectively (Teahan et al., 1991). EML I

and II differ by three amino acid residues at positions 13, 37, and 41. In this paper, we report the calcium-binding property and unfolding profile of EML I.

The echidna milk lysozyme was a kind gift from Michael Messer of The University of Sydney, Sydney, Australia. The canine milk lysozyme (CML) was purified by the method described by Pervaiz and Brew (1986) from the milk of the Pomeranian dog. Guanidinium chloride and Good buffers were of specially prepared reagent grade from biochemical studies and purchased from Nacalai Tesque Inc. (Kyoto, Japan). Quin-2 and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Apo-proteins of EML and CML were prepared by Bio-Gel P-4 column chromatography as described in a previous work (Nitta et al., 1988). The concentrations of EML and CML were determined by absorbances at 280 nm using the absorption coefficients: 20.7 and 23.2 for EML and CML, respectively, which were estimated from amino acid compositions according to the literature (Wetlaufer, 1962). The concentration of calcium ions was determined with an atomic absorption spectrophotometer (Hitachi 170-10). The change in absorbance of Quin-2 was measured with a Hitachi U2000 spectrophotometer. Circular dichroism spectra of the lysozymes were measured using a Jasco J-500 spectropolarimeter equipped with a Neslab RTE-9 circulating bath to control the sample temperature to 25 °C. A cell with an optical pathlength of 1 cm was used for measurements at 250–330 nm, and one of 0.1 cm was used for the far UV spectra (200–250 nm).

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