Journal of Biomolecular NMR, **19:** 387–388, 2001.
KLUWER/ESCOM
© 2001 *Kluwer Academic Publishers. Printed in the Netherlands.*

Letter to the Editor: Assignment of ^{1}H, ^{13}C, and ^{15}N resonances of canine milk lysozyme

Yoshihiro Kobashigawa[a,b], Kazunori Miura[a,b], Makoto Demura[b], Nobuaki Nemoto[c], Takumi Koshiba[b], Katsutoshi Nitta[b] & Sakae Tsuda[a,*]

[a]*Bioscience and Chemistry Division, Hokkaido National Industrial Research Institute (HNIRI), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan;* [b]*Division of Biological Sciences, Graduate School of Science, Hokkaido University, Kita 8 Nishi 5, Kita-ku, Sapporo 060-0808, Japan;* [c]*Varian Japan, Varian Japan Sumitomo Shibaura Bldg., 4-16-36 Shibaura, Minato-ku, Tokyo 108-0023, Japan*

Received 7 November 2000; Accepted 16 January 2001

Key words: Ca^{2+}-binding, canine milk lysozyme, chemical shift, α-lactalbumin

Biological context

The molten globule state of a protein has been extensively studied for the members of the lysozyme family (e.g., lysozyme from chicken and turkey hen egg, lysozyme from human and equine milk, and α-lactalbumin from bovine, human, goat and guinea-pig milk) (Kikuchi et al., 1998; Koshiba et al., 2000). These studies help to clarify the mechanism of the structural folding of a protein. However, its relevance to the Ca^{2+}-binding is not understood well. Canine milk lysozyme (129 amino acid residues, $M_r = 14.5$ kDa; denoted as CML) is a new member of the lysozyme family which affords to bind 1 mol of Ca^{2+} with high affinity ($k_d \sim 10$ μM) (Kikuchi et al., 1998). Study revealed that CML possesses a molten globule state at neutral pH in the presence of chemical denaturant (e.g. guanidine-hydrochloride) only when it takes a Ca^{2+}-free (apo) form. Hence, CML is regarded as a good model to provide information about the Ca^{2+}-induced effect on the molten globule state. Within the lysozyme family, equine milk lysozyme (EML) and bovine α-lactalbumin (BLA) each bind 1 mol of Ca^{2+} ion, similar to CML. The former exhibits 82% amino acid sequence homology to CML, while the latter shows only 37% sequence homology. A recent determination of the crystal structure of apo CML in the native state (Koshiba et al., 2000) revealed that the overall structural motif of apo CML is not distinguishable from that of a member of the lysozyme family; the principal structural constituents are four α-helices, three anti-parallel β-strands, and four disulfide bonds. It also revealed that a segment from Asp^{85} to Asp^{91} of CML constructs a Ca^{2+}-binding loop, whose structural motif is similar to the EF-hand (Koshiba et al., 2000). The NMR structural determination is expected to provide crucial information about the influence of Ca^{2+}-binding on molten globule formation. However, the NMR solution structure has only been determined for chicken hen egg white lysozyme (HEL) among the lysozyme family (Smith et al., 1993). Here we report the 2D- and 3D-NMR-based resonance assignments of CML in the apo- and Ca^{2+}-bound (holo) states, which will lead to determination of the detailed differences in structural construction of CML between the two states, and its relevance to the formation of the molten globule.

Methods and experiments

^{15}N- and ^{13}C/^{15}N-labeled CML with a Ser residue attached at the N-terminus was prepared as described previously (Koshiba et al., 2000). For NMR experiments, the CML sample was dissolved in H_2O containing 10% of D_2O (1–1.5 mM); the pH was adjusted to 4.5 by NaOD and DCl. 10 mM of EDTA and $CaCl_2$ were added so as to prepare the samples of apo- and holo-CML, respectively. A standard set of 2D- and 3D-NMR spectra (Cavanagh et al., 1996) was acquired for the spectral assignment of the ^{1}H, ^{13}C and ^{15}N resonances of holo-CML at 30 °C. The assignment of

*To whom correspondence should be addressed. E-mail: tsuda@hniri.go.jp

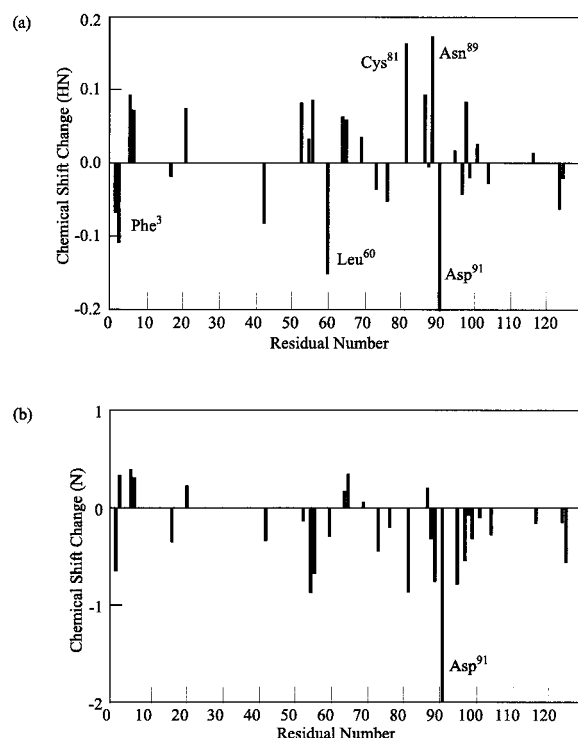

Figure 1. Plot of the chemical shift difference of canine milk lysozyme between the apo- and holo-states at 20 °C ($\delta_{apo} - \delta_{holo}$) for (a) HN-resonances and (b) ^{15}N-resonances. A large chemical shift change was observed for HN-proton (0.527 ppm) and ^{15}N (6.28 ppm) resonances of Asp^{91}.

apo-CML was carried out at 20 °C because of its low thermodynamic stability at 30 °C.

All the 2D- and 3D-NMR data were processed using NMRPipe (Delaglio et al., 1995) and XEASY (Bartels et al., 1995) software.

Extent of assignments and data deposition

Assignment of all the backbone HN- and ^{15}N-resonances of apo- and holo-CML at 20 °C was completed except for Asp^{53} and Ser^{82}. In addition, all the $^{13}C^{\alpha}$-, $^{13}C^{\beta}$-, and H^{α}-resonances of holo-CML were assigned at 30 °C except for the $^{13}C^{\alpha}$- and $^{13}C^{\beta}$-resonances of Ser^{52}, Asn^{60}, and Ser^{81}, and the $^{13}C^{\beta}$-resonances of Ser^{61} and Val^{98}. These chemical shift assignments have been deposited in the BioMagResBank database with accession numbers BMRB-4876 for holo-CML at 30 °C, BMRB-4887 for holo-CML at 20 °C, and BMRB-4883 for apo-CML at 20 °C.

It was found that the chemical shifts of the HN-resonances of apo- and holo-CML at 20 °C are almost identical with each other (within +/− 0.1 ppm) except for Phe^{3}, Leu^{60}, Cys^{81}, Asn^{89} and Asp^{91} (Figure 1). The good consistency is also identified for the ^{15}N-resonances between apo- and holo-CML, except for Asp^{91}. The residues that show large chemical shift changes between the two states (Cys^{81}, Asn^{89} and Asp^{91}) are found to be located around the Ca^{2+}-binding site of CML, suggesting that a local Ca^{2+}-induced structural change occurs in CML by the Ca^{2+}-binding. Furthermore, it appeared that the amide proton of Asp^{91} resonates at extremely low field in the holo state (10.12 ppm) and in the apo state (9.59 ppm). Such a low-field shift is also identified for the HN-resonance of the equivalent residue (Asp^{91}) of EML (9.94 ppm) (Morozova-Roche et al., 1997) and BLA (10.44 ppm) (Forge et al., 1999). Again, these two proteins can bind 1 mol of Ca^{2+} similarly to CML. In contrast, HEL and human lysozyme, which have no ability to bind Ca^{2+} (Redfield and Dobson, 1990), do not show such low-field shift of the HN-resonance (7.79 and 7.68 ppm, respectively). These data suggest that the manner of hydrogen-bonding of the amide group of Asp^{91} is highly correlated with the Ca^{2+}-binding property of the lysozyme family. A more detailed structural study on CML in the apo- and holo-states is currently in progress on the basis of the present NMR assignment.

References

Bartels, C., Xia, T.-H., Billeter, M., Güntert, P. and Wüthrich, K. (1995) *J. Biomol. NMR*, **6**, 1–10.

Cavanagh, J., Fairbrother, W.J., Palmer, A.G. and Skelton, N.J. (1996) *Protein NMR Spectroscopy*, Academic Press, San Diego, CA.

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J. and Bax, A. (1995) *J. Biomol. NMR*, **6**, 277–293.

Forge, V., Wijesinha, R.T., Balbach, J., Brew, K., Robinson, C.V., Redfield, C. and Dobson, C.M. (1999) *J. Mol. Biol.*, **288**, 673–688.

Kikuchi, M., Kawano, K. and Nitta, K. (1998) *Protein Sci.*, **7**, 2150–2155.

Koshiba, T., Yao, M., Kobashigawa, Y., Nakagawa, N., Isao, T., Kuwajima, K. and Nitta, K. (2000) *Biochemistry*, **39**, 3248–3257.

Morozova-Roche, L.A., Arico-Muendel, C.C., Haynie, D.T., Emelyanenko, V.I., Van Dael, H. and Dobson, C.M. (1997) *J. Mol. Biol.*, **268**, 903–921.

Redfield, C. and Dobson, C.M. (1990) *Biochemistry*, **29**, 7201–7214.

Smith, L.J., Sutcliffe, M.J., Redfield, C. and Dobson, C.M. (1993) *J. Mol. Biol.*, **229**, 930–944.