

# Role of the osmolyte taurine on the folding of a model protein, hen egg white lysozyme, under a crowding condition

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**Abstract** Taurine is one of the osmolytes that maintain the structure of proteins in cells exposed to denaturing environmental stressors. Recently, cryoelectron tomographic analysis of eukaryotic cells has revealed that their cytoplasm is crowded with proteins. Such crowding conditions would be expected to hinder the efficient folding of nascent polypeptide chains. Therefore, we examined the role of taurine on the folding of denatured and reduced lysozyme, as a model protein, under a crowding condition. The results confirmed that taurine had a better effect on protein folding than did  $\beta$ -alanine, which has a similar chemical structure, when the protein to be folded was present at submillimolar concentration. NMR analyses further revealed that under the crowding condition, taurine had more interactions than did  $\beta$ -alanine with the lysozyme molecule in both the folded and denatured states. We

concluded that taurine improves the folding of the reduced lysozyme at submillimolar concentration to allow it to interact more favorably with the lysozyme molecule. Thus, the role of taurine, as an osmolyte in vivo, may be to assist in the efficient folding of proteins.

**Keywords** Crowding · Lysozyme · NMR · Protein folding · Osmolyte, taurine

## Introduction

Organic osmotic solutes (osmolytes), polyhydric alcohols, free amino acids and their derivatives, and combinations of urea and methylamines are the three types of osmolyte systems found in all water-stressed organisms except the halobacteria (Yancey et al. 1982). Several investigations have explored the mechanisms by which osmolytes stabilize proteins to protect them against environmental stress (Lee and Timasheff 1981; Arakawa et al. 1990; Timasheff 1993; Kita et al. 1994).

For example, the osmolyte sucrose has been shown to induce a denatured state of hen lysozyme into a compact structure (Ueda et al. 2001) and to induce a denatured state of mutant Wil to a native-like structure (Abe et al. 2013) by increasing hydrophobic effects through preferential hydration, a mechanism first suggested by Timasheff's group (Lee and Timasheff 1981; Arakawa et al. 1990; Timasheff 1993; Kita et al. 1994). On the other hand, it has also been reported that trimethylamine-N-oxide, an osmolyte, did not affect the strength of hydrophobic interactions (Athawale et al. 2005) and had an influence on the backbone of proteins (Hu et al. 2010). These results indicate that the effects of different osmolytes are not always identical.

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Taurine is another type of osmolyte and an abundant free amino acid found in mammalian cells. Although it is one of the few amino acids not used in protein synthesis, it is one of the most essential substances in the body because of its many cytoprotective attributes (Hu et al. 2010) and its functional significance in cell development, nutrition, and survival (Sturman and Gaull 1975; Sturman 1983). In addition, taurine is widely distributed in the body, and thus early perinatal exposure to taurine frequently occurs; such exposure has been shown to have [beneficial] effects on adult arterial pressure, including cardiac effects, vascular effects and effects on the central nervous system (Roysommuti and Wyss 2014). Moreover, the potential mechanism for the suppressive effect of taurine on the pathogenesis of atherosclerosis has been discussed (Murakami 2014). In 2002, cryoelectron tomographic analysis of eukaryotic cells revealed that these cells are crowded with proteins (Medalia et al. 2002). Indeed, taurine was reported to be able to promote cell survival through its action on stress granules, since macromolecular crowding regulates the assembly of mRNA stress granules after osmotic stress (Boundedjah et al. 2012). However, since there have been few investigations into the effects of taurine on proteins at submillimolar concentration, the biological significance of taurine as an osmolyte under crowding conditions remains unclear.

Lysozyme continues to be employed as a model protein to examine the effects of molecules on protein aggregation or amyloid formation (Dubey and Kar 2014; Emadi and Behzadi 2014; Gao et al. 2013; Gazova et al. 2013). In this study, therefore, to determine the biological effects of taurine at submillimolar protein concentration in vitro, we examined the effect of taurine on the folding of hen egg-white lysozyme (hereafter, lysozyme) as a model protein and the interaction between taurine and lysozyme using multinuclear NMR.

## Materials and methods

### Materials

Five-times-recrystallized hen egg-white lysozyme was donated by the QP Company (Tokyo).

### Renaturation of lysozymes in the presence of taurine or $\beta$ -alanine

Renaturation of lysozymes was carried out according to Ueda et al. (1990) with a slight modification. Briefly, a reduction mixture was made by dissolving lysozyme (2 or 20 mg) in 1 mL of 0.584 M Tris-HCl buffer (pH 8.6) containing 8.125 M urea and 5.37 mM EDTA and reducing with fresh 2-mercaptoethanol (12.5  $\mu$ l) at 40 °C for

2 h under a nitrogen atmosphere. For the regeneration mixture, 19.8 ml of 0.1 M Tris-HCl buffer at pH 8.0 containing 1 mM EDTA and 0.66 mM oxidized glutathione in the presence of 0.2 M taurine or  $\beta$ -alanine was preincubated at 40 °C. Renaturation of reduced lysozyme was initiated by adding 200  $\mu$ l of the reduction mixture to the regeneration mixture with stirring, and was monitored by following the regeneration of the lytic activity according to the literature (Saxena and Wetlaufer 1970).

### Expression and purification of $^{15}\text{N}$ -labeled lysozyme by *Pichia pastoris*

Expression and purification of the  $^{15}\text{N}$ -labeled lysozyme was carried out according to the method of Mine et al. (1999).

### $^1\text{H}$ - $^{15}\text{N}$ HSQC NMR spectra of $^{15}\text{N}$ -labeled lysozyme in the presence of solutes

$^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra were recorded at 35 °C on a Varian Inova 600 MHz spectrometer. NMR samples (50  $\mu$ M) of lysozyme were dissolved in 50 mM acetate buffer at pH 3.8 and 10 %  $\text{D}_2\text{O}$  and were dissolved in 50 mM acetate buffer (pH 3.8) containing 0.05 M taurine, 0.2 M taurine, 0.05 M  $\beta$ -alanine or 0.2 M  $\beta$ -alanine and 10 %  $\text{D}_2\text{O}$ .

### $^1\text{H}$ - $^{15}\text{N}$ HSQC NMR spectra of $^{15}\text{N}$ -labeled denatured and S-alkylated lysozyme in the presence of solutes

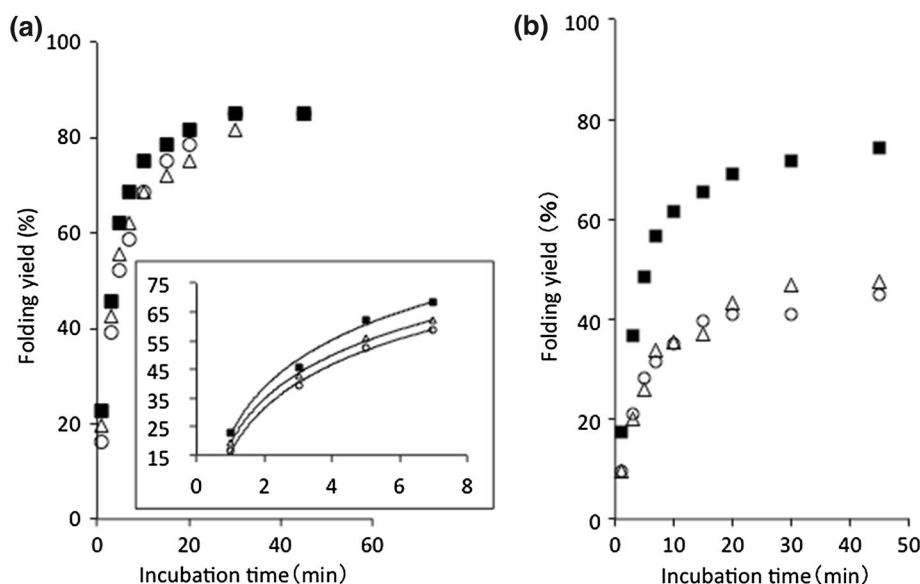
Preparation of denatured and S-alkylated lysozyme with N-(3-bromopropyl)-N,N,N',N',N'-pentamethyl-1,3-propanedi (ammonium bromide) (TAP2-Br) was carried out according to a previous paper (Yamada et al. 1994).  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra were recorded at 35 °C on a Varian Inova 600 MHz spectrometer. NMR samples (0.1 mM), which consisted of the denatured and S-TAP2 lysozyme, were dissolved in 20 mM acetate buffer (pH 6.0) and 10 %  $\text{D}_2\text{O}$  in the presence of 0.05 M taurine, 0.2 M taurine, 0.05 M  $\beta$ -alanine and 0.2 M  $\beta$ -alanine or were dissolved in 20 mM acetate buffer (pH 3.8) and 10 %  $\text{D}_2\text{O}$  in the presence of 0.05 M taurine, 0.2 M taurine, 0.05 M  $\beta$ -alanine and 0.2 M  $\beta$ -alanine.

## Results

### Effect of taurine on the renaturation of reduced lysozyme

Since the experimental conditions for the renaturation of lysozyme have been established as pH 8 and 37 °C (Ueda et al. 1990), we carried out the renaturation experiment of

**Fig. 1** The renaturation curves of reduced lysozyme at a protein concentration of 20  $\mu\text{g}/\text{mL}$  at pH 8 and 40  $^{\circ}\text{C}$  in the absence of solute (*open circles*), in the presence of 0.2 M taurine (*closed squares*) and in the presence of 0.2 M  $\beta$ -alanine (*open triangles*) are shown in panel (a). The inset indicates the early stage of the renaturation curve. The renaturation curves of reduced lysozyme at a protein concentration of 200  $\mu\text{g}/\text{mL}$  at pH 8 and 40  $^{\circ}\text{C}$  in the absence of solute (*open circles*), in the presence of 0.2 M taurine (*closed squares*) and in the presence of 0.2 M  $\beta$ -alanine (*open triangles*) are shown in panel (b)



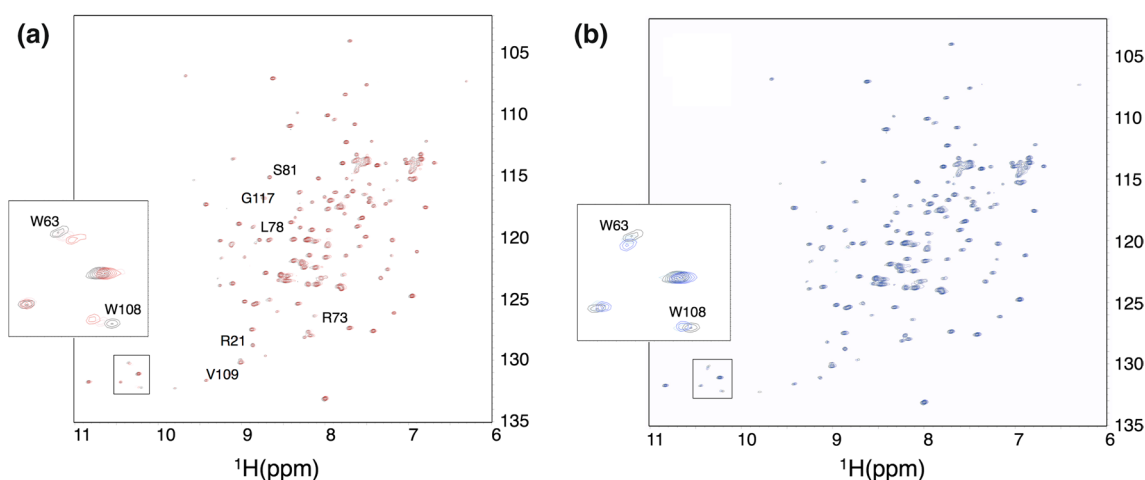
reduced lysozyme with a slight modification in the presence of taurine or, as a control, the structurally similar  $\beta$ -alanine. Figure 1a shows the renaturation curves of reduced lysozyme at a protein concentration of 20  $\mu\text{g}/\text{mL}$  in the absence or presence of 0.2 M taurine or 0.2 M  $\beta$ -alanine. The renaturation curve of reduced lysozyme in the absence of solute was almost identical to that in our previous paper (Ueda et al. 1990). About 80 % of the reduced lysozyme was renaturated to an active form under both conditions. It was found that, at the early stage of the renaturation, the folding rate of reduced lysozyme in the presence of 0.2 M taurine was slightly faster than that in the presence of 0.2 M  $\beta$ -alanine (inset in Fig. 1a). The renaturation curves from reduced lysozyme at the protein concentration of 200  $\mu\text{g}/\text{mL}$  in the presence of 0.2 M taurine or 0.2 M  $\beta$ -alanine are shown in Fig. 1b. The folding rate of reduced lysozyme was clearly faster in the presence of 0.2 M taurine than in the presence of 0.2 M  $\beta$ -alanine under this condition. Since the relationship between renaturation and aggregation from reduced lysozyme is competitive (Goldberg et al. 1991), it was found that the efficiency of taurine on the renaturation of reduced lysozyme was higher than that of  $\beta$ -alanine, whereas the yield of renaturation of reduced lysozyme at a protein concentration of 200  $\mu\text{g}/\text{mL}$  was lower than that at a protein concentration of 20  $\mu\text{g}/\text{mL}$  even in the presence of 0.2 M taurine.

#### Analysis of the interaction between taurine and lysozyme using heteronuclear NMR

As described above, when the protein was more concentrated, the effect of taurine on the renaturation of the reduced lysozyme was larger. Since NMR measurement was carried out at a protein concentration at the mM or sub-mM level, we considered that it may be appropriate

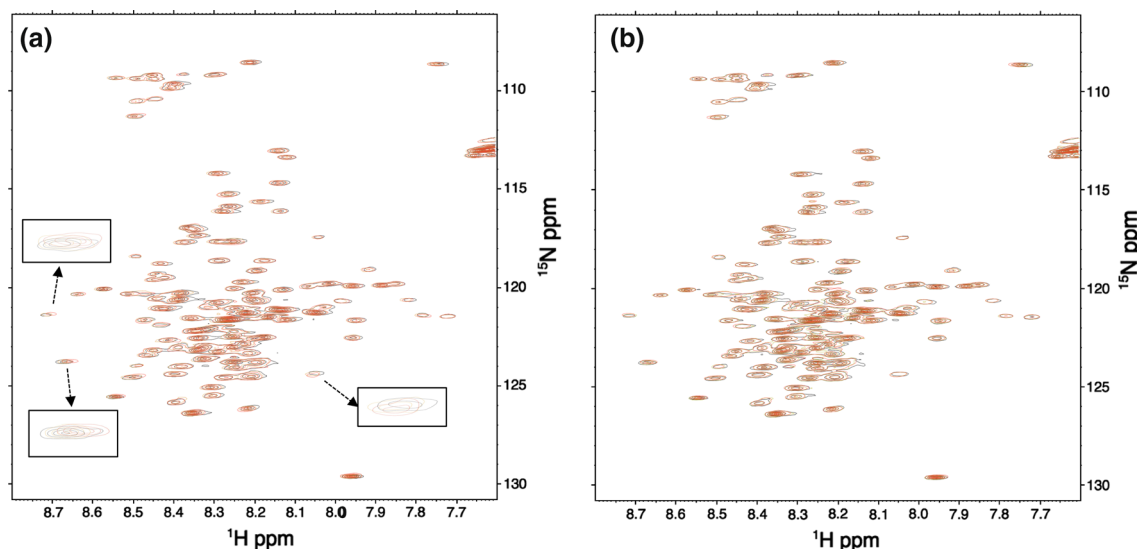
to examine the interaction between lysozyme and taurine. Moreover, since the  $^1\text{H}$  and  $^{15}\text{N}$  nuclei in lysozyme at pH 3.8 and 35  $^{\circ}\text{C}$  have been assigned (Buck et al. 1995), the interaction between lysozyme molecules and solutes have been clearly elucidated. Therefore, in our next experiment, we examined the chemical shift perturbation of cross-peaks in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of lysozyme in the presence of taurine or  $\beta$ -alanine. Figure 2a shows the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra in the absence or presence of taurine, while Fig. 2b shows  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra in the absence or presence of taurine. Although the global patterns of  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra in the presence of solutes were almost identical to those in the absence of solutes, some of the chemical shifts of cross-peaks were changed (Fig. 2a, b). When the spectra measured in the presence of taurine were compared to those in the presence of  $\beta$ -alanine, the chemical shift perturbation in  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra was slightly greater in the former. In particular, when the tryptophan residues of W63 and W108 were exposed to the solvent in the folded state of lysozyme (insets in Fig. 2a, b), it was suggested that taurine had more effect than  $\beta$ -alanine on the exposed hydrophobic residues in lysozyme.

On the other hand, the renaturation of the reduced lysozyme was carried out at pH 8. The folding of reduced lysozyme has been suggested to be competitive to its aggregation (Goldberg et al. 1991), because aggregation occurs through the denatured state. Therefore, we also examined the interactions of taurine or  $\beta$ -alanine with the denatured state of lysozyme. To characterize the denatured lysozyme, a reduced cysteine residue alkylated by cationic reagents, TAP2, can be used even at pH 8 according to a study by Yamada et al. (1994). However, the cross-peaks in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of denatured and S-alkylated



**Fig. 2** Overlaid  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of lysozyme in the absence of solute (black) or in the presence of 0.05 M taurine (thin red) or 0.2 M taurine (thick red) at pH 3.8 and 35 °C: the inset indicates the aromatic region where the chemical shift of the cross-peaks clearly changed (a). Overlaid  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of lysozyme in the absence of solute

(black) or in the presence of 0.05 M  $\beta$ -alanine (thin blue) or 0.2 M  $\beta$ -alanine (thick blue) at pH 3.8 and 35 °C are shown in panel (b). The assigned cross-peaks indicate that slight but significant chemical shift change occurred: the inset indicates the aromatic region where the chemical shift of the cross-peaks slightly changed (color figure online)



**Fig. 3** The overlaid  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of denatured and S-alkylated lysozyme in the absence of solute (black) or in the presence of 0.05 M taurine (orange) or 0.2 M taurine (red); the inset indicates the regions where the chemical shift of the cross-peaks changed (a). The overlaid  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of denatured and

S-alkylated lysozyme in the absence of solute (black) or in the presence of 0.05 M  $\beta$ -alanine (yellow-green) or 0.2 M  $\beta$ -alanine (red) is shown in panel (b). These  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of denatured and S-alkylated lysozyme were measured using 20 mM acetate buffer at pH 6.0 and 35 °C (color figure online)

lysozyme could be separated somehow when the pH of the solution was lowered to 6, at which pH most of acidic residues were dissociated and most of the amino residues were protonated in a manner similar to those at pH 8. Figure 3a, b shows the overlaid  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of denatured and S-alkylated lysozyme in the absence of solutes within the presence of 0.05 or 0.2 M taurine and 0.05 or 0.2 M  $\beta$ -alanine, respectively. In the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of denatured and S-alkylated lysozyme, the cross-peaks were

less dispersed than those of hen lysozyme in the presence of these solutes, which made it difficult to assign the cross-peaks. However, the chemical shift perturbation of cross-peaks surrounded by squares in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of denatured and S-alkylated lysozyme in the presence of taurine (Fig. 3a) was larger than that in the presence of  $\beta$ -alanine (Fig. 3b), indicating that taurine had more interactions with the denatured lysozyme than  $\beta$ -alanine did at submillimolar concentrations.

## Discussion

We showed that taurine had a greater effect than  $\beta$ -alanine on the folding of reduced lysozyme when the reduced lysozyme was more concentrated. In other words, under the condition of a concentrated amount of reduced lysozyme, the folding yield in the renaturation of reduced lysozyme was higher. The finding that taurine effectively folded the denatured and reduced protein was consistent with our previous study in which denatured and reduced humanized Fab was effectively folded (Fujii et al. 2007). Therefore, it was suggested that taurine is able to promote the folding of denatured and reduced proteins.

A number of studies have demonstrated the cytoprotective actions of taurine *in vitro* and *in vivo* (Schaffer et al. 2003). Taurine has been shown to prevent mitochondrial dysfunction and to protect against endoplasmic reticulum (ER) stress (Pan et al. 2012). In addition, taurine has been reported to ameliorate ER stress-associated diseases such as diabetes (Oprescu et al. 2007; Xiao et al. 2008) and Alzheimer's disease (Sun et al. 2014). Hypoxia, nutrient deprivation, perturbation of redox status and aberrant calcium regulation can trigger the accumulation of unfolded proteins in the ER, leading to ER stress. To counter ER stress, cells have evolved a highly conserved adaptive stress response referred to as the unfolded protein response (UPR) (Doyle et al. 2011; Ozcan and Tabas 2012). Accumulating evidence implicates ER stress-induced cellular dysfunction and cell death as major contributors to many diseases, including heart disease, diabetes, cancer and neurodegenerative diseases such as Alzheimer's and Huntington's (Doyle et al. 2011; Ozcan and Tabas 2012; Wang et al. 2014). Recently, the physiological role of taurine has been extensively studied in taurine transporter knockout mice, in which tissue taurine levels are dramatically decreased (Warskulat et al. 2007; Ito et al. 2010). These mice exhibit elevated ER-stress and accelerated cell apoptosis. Although taurine has been shown to act as a chemical chaperone in the cells, the underlying mechanisms remain to be defined. The present findings suggest that the essential role of taurine in maintaining cellular homeostasis can be explained in part by its direct effect on protein folding. In addition, this effect may be closely associated with the beneficial effect of taurine on a wide variety of disorders, including cardiovascular, metabolic and neurodegenerative diseases.

To examine the reason why taurine improved the folding of denatured and reduced proteins, we used multinuclear NMR to examine the amino acid residues in lysozyme that interacted with taurine. To our knowledge, there have been few reports on the interaction between taurine and proteins at the atomic level. In the comparison of  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra between in the presence of taurine and  $\beta$ -alanine (Fig. 2a, b), the chemical shifts of the cross-peaks

(for example, the exposed hydrophobic residues of W63 or W108) in the presence of taurine were larger than those in the presence of  $\beta$ -alanine. Since the pKa of sulfonic acid was lower than that of common carboxylic acid (Irving et al. 1980), the difference in the pKa between the sulfonic acid in taurine and carboxylic acid in  $\beta$ -alanine would have some effect on the folding efficiency and the interaction between lysozyme and a solute determined by the NMR experiment. For example, the difference should have more effect on the protonation of the amino group in taurine, resulting in the increase of cation- $\pi$  interactions between the amino group in taurine and tryptophan residues in lysozyme. Therefore, the finding that there were more interactions between taurine and the exposed hydrophobic residues in lysozyme than between  $\beta$ -alanine and the exposed hydrophobic residues in lysozyme may have been related to the improved refolding of reduced lysozyme at submillimolar concentration in the presence of taurine.

In a previous study, describing specific interactions between lysozyme and its substrate analogue, a trimer of N-acetylglucosamine, at submillimolar protein concentration (Imoto et al. 1972), some signals in the NMR spectrum became broad. However, in the present study, there were no broadening cross-peaks in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum in the presence of taurine, whereas chemical shift changes in some cross-peaks were observed (Fig. 2a). Therefore, there were no amino acid residues where taurine bound strongly to the lysozyme molecule. Instead, taurine weakly interacted with some amino acid residues in lysozyme than  $\beta$ -alanine hardly interacted with them. A similar observation was obtained by multinuclear NMR analysis of the interaction of taurine with the disordered region in both the CedA protein (at pH 5) and the PriC N-terminal domain (at pH 7.5), which are DNA-binding proteins with a globular structure (data not shown). This was consistent with the finding that taurine had more interactions with the denatured and S-alkylated lysozyme than  $\beta$ -alanine did (Fig. 3a, b). The result that taurine showed weak or no binding to amino acid residues in lysozyme was consistent with the previous result that sucrose, which is an osmolyte like taurine, bound to amino acid residues in the denatured 3mutWil protein at pH 2 (Abe et al. 2013). Since sucrose has a property of preferential hydration, taurine may have a similar ability to maintain the water around a protein by weakly interacting with the protein, resulting in efficient refolding of reduced lysozyme due to an increase in favorable hydrophobic interactions in lysozyme folding.

Zhou suggested that macromolecular crowding had an affect on the biochemical, biophysical and biophysiological properties of proteins (Zhou 2013). For eukaryotic cells, it has been suggested that most proteins appear to be solvated by only one or two water layers due to molecular crowding (Medalia et al. 2002). Thus, the preferential hydration



effect of taurine would contribute highly to the folding of denatured and reduced lysozyme at submillimolar protein concentration. Our present results that taurine may maintain the water molecules around protein molecules more efficiently than  $\beta$ -alanine and thereby improve the efficiency of protein folding would be significant in considering the osmotic effect of taurine in cells. Our findings suggest that the effect on protein folding is a basic and key action of taurine in maintaining cellular homeostasis.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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