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Opposing effects of NaCl on reversibility and thermal stability of halophilic β-lactamase from a moderate halophile, *Chromohalobacter* sp. 560

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Abstract

β-lactamase from a moderately halophilic organism is expected to show salt-dependent stability. Here we examined the temperature-dependence of stability at different salt concentrations using circular dichroism (CD) and enzyme activity. NaCl showed opposing effects on melting temperature and reversibility of the thermal melting. Increasing NaCl concentration greatly increased the melting temperature from, e.g., 41 °C in the absence of NaCl to 61 °C in 3 M NaCl. Conversely, reversibility decreased from 92% to 0% in the corresponding NaCl solutions. When β-lactamase was heated at different temperatures and NaCl concentrations, the activity recovery followed the reversibility, not the melting temperature. Heating β-lactamase at 63 °C, slightly above the onset temperature of melting in 2 M NaCl and far above the melting in 0.2 M NaCl, showed a much greater recovery of activity in 0.2 M NaCl than in 2 M NaCl, again consistent with the reversibility of melting.

Keywords: Halophilic; *Chromohalobacter*; β-lactamase; Refolding; Salt concentration

1. Introduction

Halophilic organisms are classified into essentially two groups, i.e., moderately and extremely halophilic groups. To balance the osmotic pressure of external high salts, extreme halophiles take 'salt-in' strategy incorporating high concentration of salt ions into the cells, while moderate halophiles take 'salt-out' strategy with synthesis or incorporation of organic compounds, so-called compatible solutes, in their cells [1]. Thus, intracellular salt concentration of moderate halophiles is much lower than that of extreme halophiles. Most proteins from extreme halophile adapt to and consequently require high salt concentrations for their stability; i.e., they possess high halophilicity [2]. On the contrary, proteins from moderate halophile may be grouped into two types depending on their subcellular localization. Many cytoplasmic proteins show lower halophilicity, due to low cytoplasmic salinity as described above. On the other hand, extracellular proteins, which are secreted into periplasm and external medium, must adapt to high salinity and hence show high halophilicity. It is therefore important to fully understand the influence that salt confers to individual enzymes from moderate halophiles. Here we examine the effects of NaCl on the heat stability of β-lactamase (BLA) isolated from periplasmic space of a moderate halophile, *Chromohalobacter* sp. 560.

BLA is a clinically important enzyme. More than 200 species of BLA have been isolated [3], most of which were reported to be denatured by heat treatment at 60–70 °C [4–11]. We have found previously that BLA isolated from moderate halophile (HaBLA) is stable and functional over the wide range of salt concentrations, i.e., 0∼3 M NaCl, and that its primary structure is highly abundant in acidic amino acids (pI calculated is 4.19), one of the characteristics of halophilic proteins. HaBLA showed extremely heat-resistant properties (remains ∼75% active after boiling for 5 min) in the presence of 0.2 M NaCl and that the apparent thermostability of this enzyme arises from efficient renaturation after heat denaturation [12]. Here we examine in detail the effects of salt concentrations on the thermal melting and reversibility of HaBLA. We have examined the thermal stability of the enzyme

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using temperature scan of circular dichroism (CD) signals and the enzymatic activity in the presence of NaCl at different concentrations.

2. Materials and methods

2.1. Expression, purification, and activity assay of HaBLA

Purification of HaBLA, cloning of its gene *bla* from a moderately halophile, *Chromohalobacter* sp. 560, and construction of expression vector, *pET-bla*, were described previously [12]. Recombinant HaBLA (41,830 Da) was expressed with N-terminal His-tag in *E. coli* BL21(DE3) cells harboring pET-*bla* and purified as described in previous report [12]. The enzyme activity was measured according to our previous report [12] using nitrocefin (Calbiochem) as a substrate; briefly, 0.1 mM nitrocefin and ~40 ng enzyme in 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl were incubated in temperature-controlled cuvette at 25 °C, and the amount of hydrolyzed product was monitored at 486 nm [12].

2.2. Thermal stability of enzymatic activity

The stability of enzymatic activity was studied by heating the enzyme (0.96 mg ml^{-1}, 22.95 μM) in 50 mM Tris–HCl buffer, pH 8.0, containing 0–3 M NaCl at various temperatures for 5 min and cooling it to complete equilibration on ice for 1h. Alternatively the enzyme in 50 mM Tris–HCl buffer, pH 8.0, containing 0.2 or 2 M NaCl was incubated at 63 °C as a function of time and cooled to equilibrium on ice for 1 h before measuring the residual activity.

2.3. Circular dichroism (CD) spectroscopy

CD was carried out on a Jasco J-715 spectropolarimeter using a 0.1 cm cell and a Peltier cell holder controlled by a PTC-348 temperature controller. The signal change was monitored at 217 nm with a scan rate of 0.5 °C min^{-1}, from 35 °C to about 70 °C.

2.4. Differential scanning calorimetry

The thermal stability of BLA was studied by differential scanning calorimeter (nanoDSC II, CSC, Utah, USA). BLA was dialyzed against 25 mM sodium phosphate buffer, pH 7.4, containing 0, 0.2 or 2 M NaCl at 4 °C. The dialysates were used as blank references.

Heating scans were recorded in the range of 0–70 or 80 °C at a heating rate of 1 °C min^{-1}.

After cooling the sample rapidly to 0 °C, a second run was performed.

3. Results and discussion

3.1. Thermal transition of β-lactamase

Thermal transition of HaBLA was measured by CD. The signal change at 217 nm with temperature is shown in Fig. 1 for 0, 0.2 and 2 M NaCl. The CD intensity at 35 °C is identical for these 3 samples, suggesting that the secondary structure is identical in these 3 different salt solutions. The far UV CD spectra of the protein in 0–3 M NaCl confirmed the identical structure of HaBLA independent of salt concentration (data not shown). In the absence of NaCl, melting begins at ~41 °C and ends ~51 °C with a mid-temperature of ~46 °C (Fig. 1). Above 51 °C, the CD signal levels off at about half of the initial signal remaining and does not go to zero, indicating that under the conditions, the thermally unfolded HaBLA retains some secondary structures. At 0.2 M NaCl, there is a large increase in melting temperature; melting begins at ~47 °C and ends at ~57 °C, indicating about 6 °C increase in melting temperature by 0.2 M NaCl. This sample also shows the plateau value of CD at 217 nm above 57 °C. Only a steady increase in HT (high tension voltage, equivalent to absorbance) signal is seen for these samples (Fig. 1), suggesting that aggregation is not involved in the melting process. Situation changes completely in 2 M NaCl; i.e., an abrupt increase in CD intensity and HT signal is observed at ~61 °C (Fig. 1). The increase in HT is followed by its decrease at ~62 °C. The

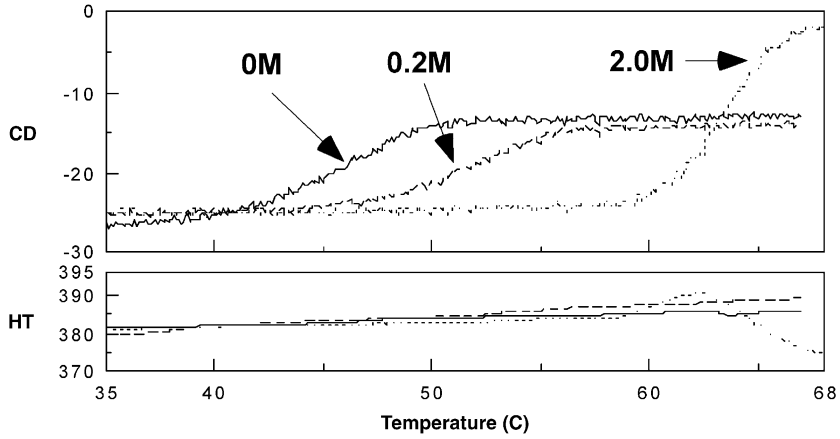

Fig. 1. Thermal transition of β-lactamase. The signal change of CD at 217 nm, expressed as ellipticity, was measured in 50 mM Tris–HCl buffer, pH 8.0 containing 0, 0.2 and 2 M NaCl. Solid line, 0 M NaCl; dash–dot line, 0.2 M NaCl; dot line, 2 M NaCl. The HT signal (equivalent to absorbance and here turbidity) increased with temperature for all three samples. All the protein samples were adjusted to 0.4 mg ml^{-1} (9.56 μM) and a cuvette with 0.1 cm pathlength was used.

increase in HT signal indicates aggregation occurring above 61 °C. The decrease of the HT signal corresponds to precipitation, i.e., proteins are moving away from the light path to the bottom of the cell. The CD signal in this case goes to zero, due to loss of the protein. It does not mean that the thermally unfolded β-lactamase in 2 M NaCl retains no secondary structure as does the protein in lower salt concentrations. It simply means that no proteins are remaining in the light path of the CD cell.

3.2. Reversibility of thermal melting

The melting data are summarized in Fig. 2. The onset temperature of melting was used to express the thermal stability of HaBLA as a function of salt concentration. Such temperature should be more appropriate when thermal melting is irreversible as for the result in 2 M NaCl. Melting temperature increases as the salt concentration is increased. Reversibility of thermal melting was examined by determining the recovery of CD signal at 217 nm after cooling the sample to room temperature for over 30 min. The CD intensity at 217 nm and 35 °C was used to calculate the recovery of the secondary structure. The observed recovery is plotted against salt concentration in Fig. 2. Reversibility is high at about 90% in 0 and 0.05 M NaCl and nearly zero in 3 M NaCl. Reversibility decreases as a function of NaCl concentration, in contrast to salt-dependent increase of melting temperature. It should be noted that the reversibility depends on the exact experimental procedure, e.g., the scan rate and end temperature of melting.

Differential scanning calorimetry (DSC) confirmed the CD results; i.e., as shown in Fig. 3, the onset of DSC endothermic peak was ~38 °C in the absence of salt, ~44 °C in 0.2 M salt and ~60 °C in 2 M salt, consistent with the values determined by CD melting. The second scan showed the majority of the heat observed in the first scan in 0 M NaCl and 0.2 M, an indication of reversibility. Little heat was observed in the second scan of the 2 M NaCl sample; i.e., the melting is irreversible, in agreement with the CD result. It appears that the peaks at low salt concentrations are not symmetric, in particular for the second scan, suggesting the presence of intermediates. It is also possible that the low salt samples have more than one conformation with different melting temperatures.

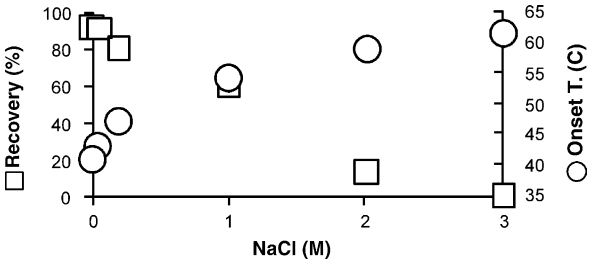

Fig. 2. Onset temperature of melting and recovery after thermal melting at various salt concentrations. Onset temperature of thermal melting and recovery of CD signal from thermal melting after cooling to room temperature were plotted at NaCl concentrations of 0, 0.05, 0.2, 1, 2, and 3 M. Circle, onset temperature of melting; Square, % recovery after melting.

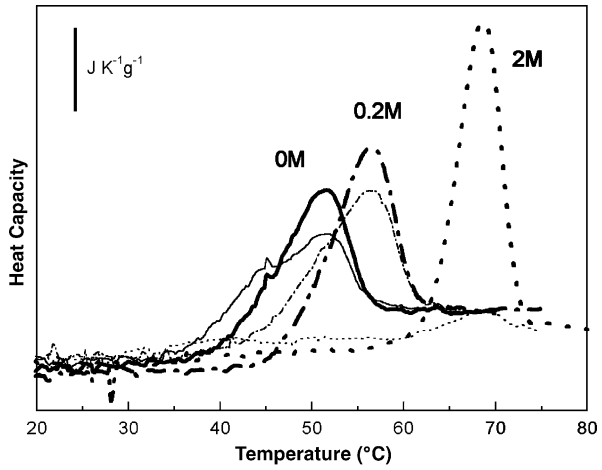

Fig. 3. DSC curves of HaBLA. DSC measurement was conducted at heating rate of 1 °C min^{-1}. HaBLA in 25 mM sodium phosphate buffer, pH 7.4, containing 0, 0.2 M or 2 M NaCl was used. The protein concentration was 4 mg ml^{-1} (95.63 μM). Thick lines represent first heating, while thin lines show second heating after cooling. Solid line, 0 M NaCl; dash–dot line, 0.2 M NaCl; dot line, 2 M NaCl.

3.3. Heat stability of enzymatic activity

Heat stability of HaBLA was also examined by enzymatic activity. The enzyme in 50 mM Tris–HCl buffer, pH 8.0, containing 0–3 M NaCl was heated at different temperatures for 5 min and cooled to equilibrium. The enzyme activity after cooling is plotted against temperature in Fig. 4. At or below 50 °C of heating, all the samples regained nearly full activity. This temperature is above the onset temperature of melting for 0–0.2 M NaCl samples; i.e., HaBLA at these salt concentrations is denatured at 50 °C. Nevertheless, they are nearly fully active after cooling, consistent with the CD data that thermal melting is highly reversible in 0–0.2 M NaCl. The samples in 1–3 M NaCl were also fully active after heating at 50 °C for 5 min and cooling, consistent with the fact that their melting temperature is above 54 °C. The 1 M sample starts to melt at

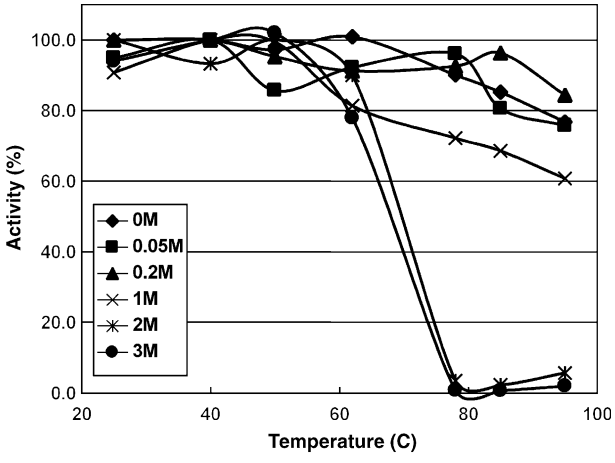

Fig. 4. The residual activity after heating to the indicated temperature for 5 min and cooling on ice to equilibrium. The HaBLA (0.96 mg ml^{-1}, 22.95 μM) was heat-treated at the temperature shown for 5 min in 50 mM Tris–HCl buffer, pH 8.0, containing 0–3 M NaCl and residual activity after cooling on ice for 1 h was measured as described in Section 2.

~54 °C, close to the temperature used, yet shows a full recovery of enzyme activity. The reversibility of thermal melting in 1 M NaCl is only 60%, suggesting that the incubation time of 5 min may be too short to cause any irreversibility at 50 °C. Heating above 50 °C resulted in varying degree of activity loss. Heating at 62–85 °C showed high activity recovery for 0–0.2 M NaCl samples, despite the high temperature used; these temperatures are far above the melting temperatures of HaBLA in 0–0.2 M NaCl. This indicates that heating of these samples at 62–85 °C causes no additional changes in the unfolded structure and hence confers to them an ability to regain the native structure upon cooling. However, heating at 95 °C for 5 min appears to cause some irreversible change in the protein in 0–0.2 M NaCl, as indicated by the partial loss of activity.

Heating at 62–95 °C caused varying degree of activity loss for 1–3 M NaCl samples. Heating at 62 °C resulted in partial loss of activity even for 3 M NaCl sample, whose melting (its onset melting temperature of 61 °C) begins around this temperature. This is consistent with the fact that the thermal unfolding of HaBLA is completely irreversible in 3 M NaCl. It is interesting that the 1 M sample recovers more activity than does the 2 or 3 M NaCl sample, although its melting temperature is the lowest (54 °C), far below the heating temperature of 62–95 °C. This indicates the importance of reversibility, not melting temperature, for regaining the enzyme activity after high temperature treatment.

3.4. Time course of residual enzymatic activity incubated at 63 °C in the presence of 0.2 or 2 M NaCl

Lastly we have examined the effect of incubation time at heating temperature of 63 °C for 0.2 and 2 M NaCl. The HaBLA was incubated for indicated period of time in Fig. 5 at 63 °C, slightly above the onset temperature of melting in 2 M NaCl and far above the end temperature of melting in 0.2 M NaCl. The sample in 0.2 M NaCl slowly lost activity recovery with incubation time to a level of ~80% after 180 min of heating, indicating that heating at 63 °C of the thermally unfolded HaBLA slightly alter the reversible nature of unfolded structure in 0.2 M NaCl. Conversely, heating of the 2 M NaCl sample caused the loss of activity with time to nearly no activity at 60 min incubation. Since this temperature is only slightly above the onset temperature of melting, the results suggest that as soon as the protein unfolds in high salt, it leads to irreversibility consistent with the reaction order; i.e., it appears that the plot of log (residual activity) vs. time for the 2 M data shows a linear relation, suggesting that the reaction is first-order and hence the rate limiting step is unfolding.

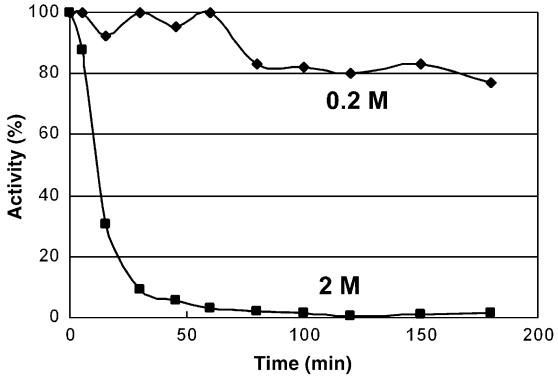

Fig. 5. Time course of residual activity incubated at 63 °C. The HaBLA (0.33 mg ml^{-1}, 7.89 μM) was incubated for indicated period of time at 63 °C in 50 mM Tris–HCl buffer, pH 8.0, with 0.2 or 2 M NaCl and measured residual enzymatic activity after cooling on ice for 1 h.

There are two explanations for the observed irreversibility. First one is that the native to unfolded conformational transition is irreversible. In this case, the observed reversibility or partial reversibility reflects the amount of the native protein remaining after thermal unfolding. The second possibility is a two-step unfolding; i.e., reversible thermal unfolding followed by a reaction leading to irreversible products. In the latter case, NaCl increases the kinetics of the second reaction. If the irreversible reaction is due to aggregation and the rate limiting step, then increasing protein concentration should enhance the irreversible process, inconsistent with the reaction order described above.

What is the mechanism of the effects of NaCl on melting temperature and irreversibility? NaCl is a weak salting-out salt and protein-stabilizer. The observed increased melting temperature could be due at least in part to its general stabilizing effects. But the observed magnitude is much higher than the general stabilizing effects of NaCl and is due to specific character of halophilic protein, i.e., binding of ions to the native structure, which should further stabilize the protein. The observed effect of NaCl on reversibility is most likely due to its effect as a weak salting-out. NaCl is classified as the intermediate in the Hofmeister series. Salting-in salts, such as $MgCl_2$, and salting-out salts, such as $MgSO_4$, may be expected to change both melting temperature and reversibility. They would bind to this halophilic enzyme differently from NaCl binding and exert their general salt effects on protein stability and aggregation. Both protein concentration and salt-type dependence of the melting and reversibility of BLA thermal unfolding would further clarify the mechanism of the salt effects and such study is underway.

In contrast to extremely halophilic bacteria, which are unable to survive and tend to lyse at salt concentrations below 2.5 M, moderately halophilic bacteria can grow over a wide range of salinities: optimally at 0.5–2.5 M salts, but usually at much wider conditions. Thus, moderately halophilic bacteria in natural habitats frequently encounter with sudden changes in environmental salinity, and the periplasmic proteins must be adaptable to such fluctuation of salt concentrations. This HaBLA shows reasonable properties to maintain its physiological activities in wide range of salinity conditions, i.e., higher reversibility in lower salt conditions and higher conformational stability in higher salt conditions. It should be noted that reversibility and high melting temperature are different concept. In low salt conditions, additional stress, e.g., high temperature, may cause denaturation and loss of activity due to instability, but upon its removal, the protein

returns to the native state. Conversely, high melting temperature in higher salt conditions renders the protein active even at such conditions, although long-term stability is compromised due to irreversibility.

4. Conclusion

We have shown here that NaCl shows opposing effects on HaBLA; i.e., it increases the melting temperature and decreases the reversibility of thermal unfolding. Binding of NaCl to the HaBLA, that should occur as observed with other halophilic proteins [13,14], does stabilizes the native structure, but makes thermal unfolding less reversible when exposed to high temperature. It is likely that the unfolded structure of HaBLA has higher tendency to aggregate in high salt than in low salt, rendering the loss of reversibility upon cooling.

Acknowledgements

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References

[1] M.I. Calderon, C. Vargas, F. Rojo, F. Iglesias Guerra, L.N. Csonka, A. Ventosa, J.J. Nieto, Complex regulation of the synthesis of the compatible solute ectoine in the halophilic bacterium *Chromohalobacter salexigens* DSM 3043T, Microbiology 150 (2004) 3051–3063.

[2] D. Madern, C. Ebel, G. Zaccai, Halophilic adaptation of enzymes, Extremophiles 4 (2000) 91–98.

[3] K. Bush, G.A. Jacoby, A.A. Medeiros, A functional classification scheme for beta-lactamases and its correlation with molecular structure, Antimicrob. Agents Chemother. 39 (1995) 1211–1233.

[4] X. Wang, G. Minasov, B.K. Shoichet, Noncovalent interaction energies in covalent complexes: TEM-1 beta-lactamase and beta-lactams, Proteins 47 (2002) 86–96.

[5] B.M. Beadle, S.L. McGovern, A. Patera, B.K. Shoichet, Functional analyses of AmpC beta-lactamase through differential stability, Protein Sci. 8 (1999) 1816–1824.

[6] H. Iwai, A. Pluckthun, Circular beta-lactamase: stability enhancement by cyclizing the backbone, FEBS Lett. 459 (1999) 166–172.

[7] G. Feller, Z. Zekhnini, J. Lamotte Brasseur, C. Gerday, Enzymes from cold-adapted microorganisms. The class C beta-lactamase from the antarctic psychrophile Psychrobacter immobilis A5, Eur. J. Biochem. 244 (1997) 186–191.

[8] M. Vanhove, S. Houba, J. Lamotte Brasseur, J.M. Frere, Probing the determinants of protein stability: comparison of class A beta-lactamases, Biochem. J. 308 (1995) 859–864.

[9] H.M. Lim, J.J. Pene, R.W. Shaw, Cloning, nucleotide sequence, and expression of the Bacillus cereus 5/B/6 beta-lactamase II structural gene, J. Bacteriol. 170 (1988) 2873–2878.

[10] A.M. Pastorino, D. Dalzoppo, A. Fontana, Properties of Sepharose-bound beta-lactamase from *Enterobacter cloacae*, J. Appl. Biochem. 7 (1985) 93–97.

[11] R.B. Davies, R.E.P. Abraham, Comparison of beta-lactamase from Bacillus cereus 569/H/9 with a beta-lactamase from Bacillus cereus 5/B/6, Biochem. J. 145 (1975) 409–411.

[12] H. Tokunaga, M. Ishibashi, T. Arakawa, M. Tokunaga, Highly efficient renaturation of beta-lactamase isolated from moderately halophilic bacteria, FEBS Lett. 558 (2004) 7–12.

[13] G. Zaccai, G.J. Bunick, H. Eisenberg, Denaturation of a halophilic enzyme monitored by small-angle neutron scattering, J. Mol. Biol. 192 (1986) 155–157.

[14] G. Zaccai, E. Wachtel, H. Eisenberg, Solution structure of halophilic malate dehydrogenase from small-angle neutron and X-ray scattering and ultracentrifugation, J. Mol. Biol. 190 (1986) 97–106.