NISR RESEARCH GRANT
2003 Research Grant

Development of Highly Soluble Enzymes Adopting the Structural Characteristics of Halophilic Enzymes

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I. Research aims:

Some specific microorganisms can grow under extreme environmental conditions, such as high temperature, extreme pH and high salt concentrations. We have attempted to characterize halophilic enzymes, aiming at expansion of their industrial applications. Extremely halophilic archaea and moderately halophilic bacteria require NaCl above 2.5 M and 0.5 M for growth, respectively and accumulate high concentrations of ions and compatible solutes inside the cells. These enzymes can function under the extreme conditions where most of the "normal" enzymes cannot, which makes industrial applications of these enzymes very attractive. The strategy of halophilic enzymes to adapt high salt conditions is a large excess content of acidic amino acids in their amino acid composition. These acidic amino acid residues are localized on the protein surface and contribute to bind excess amounts of water molecules and cationic ions. We have studied the properties of nucleoside diphosphate kinases (HsNDK) from extremely halophilic archaea, *Halobacterium salinarum*, and moderately halophilic bacteria, *Halomonas* sp., and β-lactamase (BLA), porin, and HrdC from moderately halophilic bacteria. We propose that the highly acidic molecular nature of halophilic proteins generates a high solubility without aggregation, and ensures the several interesting characteristics of halophilic proteins, such as highly efficient renaturation after denaturation treatment. We are now developing the applications of this interesting "halophilic nature" to microbiological industries and molecular biology.

II. Methods & Results

1. Nucleoside diphosphate kinase (HsNDK) from extremely halophilic archaea, *H. salinarum.*

Most halophilic enzymes isolated from extremely halophilic archaea generally require at least 1～2 M NaCl for their activity and stability. However, we have observed for the first time that HsNDK isolated from *H. salinarum* is stable and active without NaCl and then cloned *ndk* gene for its further characterization. Its amino acid composition deduced from the nucleotide sequence indicates high content of acidic amino acid residues, typical characteristics of halophilic proteins. The *ndk* gene was cloned into *E. coli* expression vector (pET3a) and was expressed in *E. coli*. The HsNDK protein expressed in *E. coli* was localized in soluble fraction, but in an inactive form. This inactive HsNDK was activated (folded) properly by dialysis against 4 M NaCl, and once folded under high salt conditions, HsNDK was stable and active even without NaCl, having the same properties as the native enzyme.

The native enzyme purified from *H. salinarum* was denatured by heat-treatment at 90°C or 6 M urea, and its renaturation (refolding) was examined in the presence of various solutes, such as salts and a compatible solute, trimethylamine N-oxide (TMAO). Denatured HsNDK was refolded in the presence of either high concentration of salts or TMAO. These results indicate that HsNDK does not require high salt concentrations for its stability and activity, but requires high salts for its native refolding. TMAO is not an electrolyte, meaning that the presence of concentrated salts is not an absolute requirement and hence that charge shielding or ion binding is not a sole factor for the folding and activation of HsNDK. TMAO is a strong protein structure stabilizer and forces the conformation equilibrium to a more compact structure, suggesting that increasing hydrophobic interactions alone can induce refolding of HsNDK.

To further characterize this enzyme, we have attempted to express HsNDK in *E. coli* with hexa-His-tag. When an extra sequence containing His-tag (derived from pET15b vector) was fused to the N-terminus of HsNDK, unexpectedly the fusion resulted in a fully active enzyme with increased expression. This is a first observation of active folding of halophilic enzyme from extremely halophilic archaeon in *E. coli*. Several lines of evidence suggest that positive charges in fused N-terminal extension are effective in suppressing the negative charge repulsion of halophilic enzyme and thus, facilitate folding and assembly of HsNDK. These observations open a prospect that small modification of halophilic protein structures can change their halophilic properties, and thus generate novel molecules from halophilic enzymes for biological industries.

2. Highly efficient renaturation of β-lactamase isolated from moderately halophilic bacteria.

Most β-lactamases reported to date are irreversibly denatured at 60～70°C. Here, we found that a halophilic β-lactamase from the moderately halophilic bacterium *Chromohalobacter* sp. 560 was highly stable against heat-inactivation: it retained ～75% of its activity after boiling for 5 min in the presence of 0.2 M NaCl, suggesting that the protein either incompletely denatures during the boiling process or readily renatures upon cooling to the assay temperature. Circular dichroism showed a complete unfolding at 60°C

and its full reversibility, indicating that the observed activity after boiling is due to efficient refolding following heat-denaturation. The gene *bla* was cloned, and the primary structure of the enzyme deduced from the gene sequence showed highly abundant acidic amino acid residues, one of the characteristics of halophilic proteins. These observations suggest that the highly acidic molecular nature of halophilic proteins generates a high solubility without aggregation, and ensures highly efficient renaturation after heat or urea treatment. We are now developing an "application" of this protein to molecular biology.

III. References

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