ABSTRACT

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Title: **Effect of Macromolecular Crowding on Ribonuclease-A Stabilization By Sugar Osmolytes.**

A major difference between the *in-vitro* folding experiments and the *in-vivo* cellular environment is the high concentration of macromolecules which include proteins, nucleic acids, ribosomes, and carbohydrates. The estimated concentration of macromolecules present in the cytoplasm is in the range of 80–400 mg/ml. This means a large fraction of the interior space of a cell is not available to other macromolecular species. Proteins are known to perform their specific tasks in the cellular environment made of high concentration of macromolecules. Macromolecular crowding involves nonspecific force of steric repulsions on particular reactions that occur in highly volume-occupied media. According to the excluded volume theory, any reaction that increases the available volume will be stimulated by macromolecular crowding. Crowding conditions can be mimicked experimentally by adding inert synthetic or natural macromolecules, termed crowding agent, to the *in-vitro* systems.
It is established now that effect of macromolecular crowding on biological macromolecules of interest may be studied experimentally by using concentrated solution of crowding agents such as dextran, ficoll, polyethylene glycol (PEG) or inert proteins.
In the present study, we have chosen dextran 70 as a macromolecular crowding agent. It has no interaction with protein apart from stearic repulsion, does not aggregate and has high solubility in physiological solution. Our observation on the effect of dextran 70 is in agreement with earlier reports that macromolecular crowding increases stabilization of various proteins. As indicated by the excluded volume hypothesis, one of the significant impacts of crowding agent on macromolecules is to destabilize the unfolded state. Excluded volume physically lessens the conformational entropy i.e., the space accesible for protein conformational fluctuations. These stearic hinderences because of crowding lead to the destabilization of unfolded state of the protein which accordingly favours the folded state by shifting the denaturation equilibrium, N (native) ↔ D (denatured) conformation, towards the N state. Thus, increases the Gibbs free energy change associated with denaturation equilibrium in the presence of crowding agent because denatured molecules are converted into the native molecules. To investigate the effect of pH on the extent of stabilization of RNase A in the presence of dextran 70, we plotted % $\Delta\Delta G_D^o$ as a function of pH. The stabilizing effect of dextran 70 was found to be increasing with the decrease in pH values from 7.0 to 2.0. This may be due to the presence of insignificant number of denatured molecules at physiological pH.

On the other hand, low molecular weight organic compounds (osmolytes) used by the cells to counteract the external stress. These osmolytes are known to stabilize the native protein structure and rescue defective proteins from proteotoxic intracellular environment.

In the past few decades, the effect of osmolytes on protein folding in dilute solutions has been well documented. In this study, we have investigated the effect of sugar osmolytes (on the basis of their sizes) on the thermodynamic stability and enzyme activity of a model protein, ribonuclease A (RNase A) in macromolecular crowded system. We have used 300 mg/ml dextran 70 to mimic the macromolecular crowding conditions as the intracelluar concentration of biological macromolecules ranges from 80-400 mg/ml. As predicted by the excluded volume theory, the estimates of equilibrium and reaction rate set up via dilute solutions vary by orders of magntitude from estimates found on the same reaction performed in macromolecular crowded conditions. We have carried out thermal denaturation measurements of RNase A in the absence and presence of six sugars (glucose, fructose, galactose, sucrose, raffinose and stachyose) in the macromolecular crowded environment in the pH range of 7.0-2.0.

The stabilizing effect of sugar osmolytes in the crowded environment is found to be size dependent with the stabilizing effect increases with the increase in the size of osmolyte. The order of stabilization is as follows: stachyose > raffinose > sucrose > glucose, fructose and galactose. The extent of stabilization of RNase A in the presence of cosolutes is pH dependent, that is, it increases with the decrease in pH from 7.0 to 2.0. The observed effect of sugar osmolytes in the presence of macromolecular crowded environment that consists of 300 mg/ml dextran 70 is equal to the sum of the effect of sugar osmolytes and dextran 70 alone. We found a gradual increase in the secondary structure of RNase A in the presence of different concentrations of dextran 70.

The crowding agent or sugar osmolytes in the crowded environment have not shown any effect on the tertiary structure as well as the heat denatured state of RNase A.

We have measured the kinetics of enzyme reactions in the presence of different concentration of the macromolecular crowding agent (dextran 70), sugar osmolytes and in the presence of sugar osmolytes in the crowded environment. We found that there is a decrease in the K_{cat} and K_m values with the increasing concentration of dextran 70. At lowest concentrations of all sugar osmolytes, we found a slight change in the values of K_{cat} and K_m. However, at higher concentrations, sugar polyols have shown to decrease the kinetic parameters, K_m and k_{cat} of the protein. Moreover, the decreasing effect on the K_{cat} and K_m values increases with the increasing size of the osmolytes from monosacchrides to tetrasaccharides. After investigating the effect of sugar osmolytes in dilution solution, we further studied the effect of sugar osmolyes in the crowded environment that consists of 300mg/ml dextran 70. We found a considerable decrease in the K_{cat} and K_m values of RNase A in the presence of all sugar osmolytes in the crowded environment. The effect of osmolyte is more in the crowded environment than the dilute environment.