Macromolecular Crowding-Induced Molten Globule State of Acid-Denatured Horse Cytochrome *c*

Thesis Submitted

In partial fulfillment of the requirement for the degree of

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CHEMISTRY

Submitted by:

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JULY, 2014

Certificate

*This is to certify that the thesis entitled "**Macromolecular Crowding-Induced Molten Globule State of Acid-Denatured Horse Cytochrome c**" being submitted in the partial fulfilment of requirements for the award of degree of **Master of Science in Chemistry** submitted in **the School of Chemistry and Biochemistry, Thapar University, Patiala** is a bonafide work carried under the supervision of **Dr.Rajesh Kumar,** Associaie Professor, School of Chemistry and Biochemistry, Thapar University, Patiala and that no part of this project has been submitted for the award of any other degree.*

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This is to certify that above statement made by the student concerned is correct and true to the best of my knowledge.

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CANDIDATE'S DECLARATION

I hereby declared that the work presented in this thesis entitled ***"Macromolecular Crowding-Induced Molten Globule State of Acid-Denatured Horse Cytochrome c"*** *submitted in the partial fulfilment of requirements for the award of degree of* ***Master of Science in Chemistry*** *submitted in* ***the School of Chemistry and Biochemistry, Thapar University, Patiala*** *is an authentic record of my own work carried out under the supervision and guidance of* ***Dr. Rajesh Kumar,*** *Associate Professor, School of Chemistry and Biochemistry, Thapar University, Patiala and refers other researcher's work which are duly listed in the reference section.*

The matter embodied in this thesis has not formed the basis for the award of any other degree of this or any other university.

Date: 18 July, 2014

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In the end, I thank our God, for giving me the intellect to understand the complexity of numbers, and giving me strength to complete this thesis.

Date: 18-July-2014

Regards,

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ABSTRACT

This work examined the effect of concentration and size of crowding agents (dextran 40 and dextran 70) on structure, stability and folding of acid-denatured ferricytochrome c at pH 2.0. As [dextran] is increased in the UA state , the intensity of flouroscence emission spectra decreases, which indicates molecular compaction of UA state. The crowding agents stabilize and refold the acid-denatured ferricytochrome *c* (pH 2.0) to MG-states. The acid-denatured ferricytochrome *c* also gains substantial secondary structure content but not acquire tertiary structure content in the presence of high concentrations of crowding agent.

1.0 Introduction

Protein folding is a physical process where a newly synthesized chain of amino acids transform to a perfectly folded functional protein under highly crowded cellular environment [1]. Pioneering experimental and theoretical work shows that protein folding dynamics are significantly influenced by macromolecular crowding conditions similar to those likely exist in vivo [2-18]. Increasing evidence indicates that macromolecular crowding in vitro significantly increases the rate of amyloid formation in several fibril forming proteins [2-3, 19-21] and play a significant role in promoting the onset of several neurodegenerative diseases [22].

The proteins can denature at extreme acidic or basic pH conditions due to charge repulsion. Increasing evidence indicates that the acid or base-denatured proteins in the presence salts, alcohols (HFIP, TFE), polyanions, and low concentrations of guanidine hydrochloride collapsed to molten globule states (MG-state) [23-27, 28]. The MG-states are classified as a third thermodynamic state of protein molecules, the native and unfolded states are the other two states [29]. MG-states generally have a substantial amount of secondary structure (*i.e.*, like native-states) but lack tertiary interactions [30-31]. The MG-states resemble the intrinsically disordered proteins (IDP) that lack or have disordered tertiary interactions [32-33].

In few earlier studies, the effect of sugars on the conformation of several proteins has been investigated and suggested that volume exclusion of high concentrations of sugars can transform the acid-denatured protein to MG-state [34-36]. Few earlier reports have also shown that the high concentrations of dextran 40 also transform the pH-denatured proteins to MG-states. While many studies have addressed the stabilizing effects of macromolecular crowding on folded and partially unfolded proteins, most of these studies have focused on only one crowding agent at a time [37]. The aim of this thesis is to systematically investigate the effects of

crowding agents with varying chemical properties and sizes at different concentrations on the structure, stability and folding of acid-denatured ferricytochrome *c*. The current results show that the high concentrations of crowding agents (dextran 40 and dextran 70) stabilize and refold the acid-denatured ferricytochrome *c* (pH 2.0) to MG-state. Interestingly, the extent of stabilization of acid-denatured to MG-state is found more for the smaller size crowding agent (dextran 40) than that of the larger size crowding agent (dextran 70). Understanding of how acid-denatured ferricytochrome *c* folds in different crowded environments is very important since it is a key protein involved in electron transfer reactions in mitochondria, 21 as well as a signaling molecule for apoptosis in the cytoplasm.

2.0 Materials and Methods

Horse heart cytochrome *c* (type VI) and crowding agents (dextran 40 and dextran 70) were purchased from Sigma Aldrich. GdnHCl and sodium sulfate were purchased from USB and sigma, respectively. All experiments were performed in 10 mM glycine buffer at pH 2.0.

2.1 Measurement of pH–equilibrium titration of ferricytochrome *c*

For pH-equilibrium titration of ferricytochrome *c*, ~8.0 μM solution of ferricytochrome *c* prepared in a mixture of 10 mM sodium acetate buffer and 10mM glycine buffer were titrated to different pH values in the 1.5-7.0 range by the use of concentrated HCl in the absence and presence of 300 mg/mL dextran 40 or dextran 70 or 1.0 M Na_2SO_4. Flouroscence emission spectra (ex: 280 nm, em: 360 nm, Slit size: 5/10) of all the samples were taken on Perkin Elmer LS 55 fluorescence spectrometer after an equilibration of the samples at room temperature for an

hour. pH of the samples was measured before and after the experiments and the reported values are those measured after the experiment. pH titration curves were analyzed by using the Henderson-Hasselbalch equation,

$$y = c_d + c_n [10^{n(pH - c_m)}]/1+10^{n(pH-c_m)} \quad (1)$$

where, c_d and c_n are normalized fluorescence signals for the denatured and native states, respectively, n is the number of H^+ titrated, and c_m is the pH midpoint for the transition.

2.2 Measurement of fluorescence and far-UV CD spectra of ferricytochrome *c* in the absence and presence of dextran 40 or dextran 70 or Na_2SO_4

Samples of ferricytochrome *c* were prepared in 10 mM glycine buffer in the absence and presence of ~300 mg/mL dextran 40 or dextran 70 or 1.0 M Na_2SO_4 at pH 2.0. The samples were equilibrated for an hour at room temperature at 25 ºC. The fluorescence and CD spectra were collected on Perkin Elmer LS 55 spectrophotometer (ex: 280 nm and em: 365 nm) and JASCO J-710 spectropolarimeter (250-190 nm), respectively at 25 ºC. Final concentrations of protein were ~ 8.0 µM and 15 µM in fluorescence and far-UV CD experiments, respectively.

2.3 Measurement of GdnHCl-induced unfolding of acid denatured ferricytochrome *c*. Fluorescence-monitored GdnHCl-induced unfolding titrations of acid-denatured ferricytochrome *c* in the 0-6.0 M range of GdnHCl were carried out in the absence and presence of ~300mg/mL of dextran 40 or dextran 70 or 0.5 M Na_2SO_4 at pH 2.0. Final concentration of protein was 9.0 µM. GdnHCl concentrations were determined by refractive index measurement on an Abbe's refractrometer. The fluorescence emission spectra were collected on Perkin Elmer LS 55

spectrophotometer at 25 ºC (ex: 280nm and em: 365nm). GdnHCl concentration and pH of the samples are those measured after the experiments.

3.0 Results

3.1 Molecular compaction of the acid unfolded ferricyt *c* in the presence of crowding agents

Fig. 1a shows the pH-titration of ferricytochrome *c* collected between pH 7.0 and pH 2.0. Clearly at pH~ 2.0, there is a substantial increase in the fluorescence intensity of the protein at pH ~2.0, which is due to structural unfolding of the protein at pH ~2.0 due to charge repulsion. pH titration of ferricytochrome *c* was analyzed by using the equation (1), which provides the pH-midpoint, $c_m \sim 2.84$ and the number of H^+ titrated, $n \sim 3.0$. Fig. 1b shows the different states of ferricytochrome *c*. Due to the lone tryptophan (W59), the fluorescence emission intensity gives a reliable indicator for the molecular compactness of ferricytochrome *c*. Because of excitation energy transfer from W59 to the heme, native ferricytochrome *c* is fluorescence-silent (solid line) [38-39]. At pH 2, upon unfolding of protein, the distance of the heme group from the W59 increases. This result in an increase in fluorescence quantum yield of the protein (short dash line). The acid-denatured ferricytochrome *c* (pH 2.0) in the presence of salt or crowding agents could possibly be stabilized and transformed to the MG-state for structural characterization. Fig. 1b clearly shows that the inclusion of 300 mg/ml dextran 40 or dextran 70 or ~1.0 M Na_2SO_4 cause the molecular compaction of acid-denatured ferricytochrome *c*.

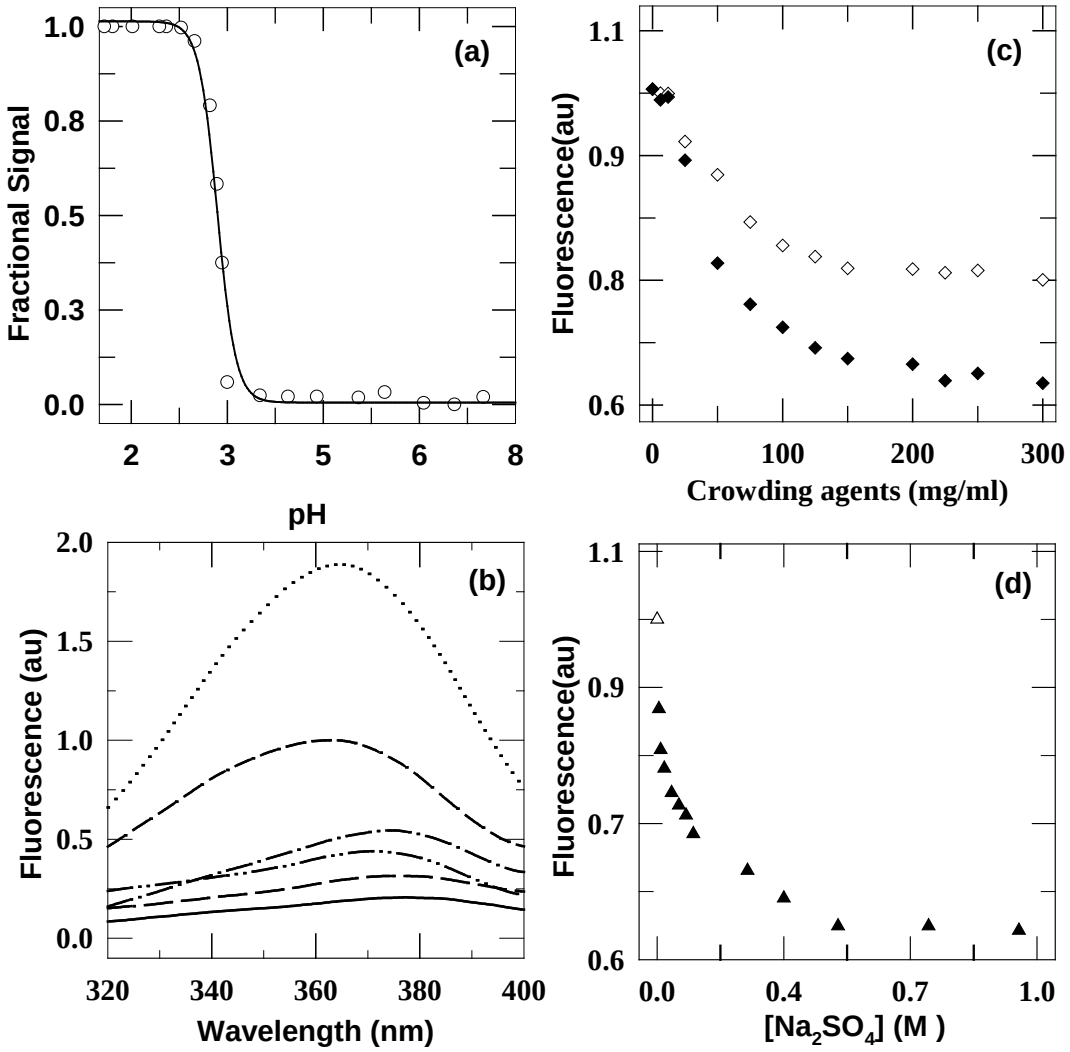

Fig. 1. Panel (a) presents the fluorescence-monitored pH-induced unfolding transitions of ferricytochrome *c*, 25°C. The solid lines are the fit of the data to Henderson-Hasselbalch equation. Panel (b) presents fluorescence spectra for different states of ferricytochrome *c*: pH 7.0, native state (solid line); GdnHCl (5.0 M) unfolded state (pH 7.0) (dotted line); acid-denatured state of ferricytochrome *c* (pH 2.0) (short dash line); 300mg/ml dextran 70 induced MG-state of acid-denatured ferricytochrome *c* (dash single dot line); 300mg/ml dextran 40 induced MG-state of acid-denatured ferricytochrome *c* (dash double dot line), and 1.0 M Na_2SO_4 induced MG-state of acid-denatured ferricytochrome *c* (long dash line) (c) Relative quenching of Trp59 fluorescence for acid-denatured ferricytochrome *c* (pH 2.0) as a function of dextran 40(♦) and dextran 70 (◊). (d) Relative quenching of Trp59 fluorescence for acid-denatured ferricytochrome *c* (pH 2.0) as a function of Na_2SO_4.

Fig. 1c shows the effect of concentration and size of crowding agent on molecular compactness of acid-denatured ferricytochrome *c* at pH 2.0. Clearly, among the dextran 40 and dextran 70, the decrease in fluorescence intensity of the acid denatured ferricytochrome *c* is more

pronounced in the presence of dextran 40 than in the presence of dextran 70. These findings indicate that the small size crowding agents results in a greater compaction of the acid denatured protein than that of the larger size crowding agents. Fig 1d shows that the relative W59 quenching of acid denatured ferricytochrome *c* in the presence of 1.0 M Na_2SO_4.

3.2 Acid-denatured ferricytochrome *c* gains substantial secondary structure in the presence of crowding agents

MG-states have substantial content of secondary structure, often comparable to that in the native states. To test whether acid-denatured ferricytochrome *c* gains substantial secondary structure in the presence of crowding agents, the far-UV CD spectra of acid denatured-ferricytochrome *c* were collected in the absence and in the presence of 400 mg/ml dextran 40, dextran 70 and 1.0 M Na_2SO_4 at pH 2.0 (Fig. 2a). Clearly, the inclusion of ~ 400 mg/ml dextran 40 or dextran 70 results in increase in CD-222 nm ellipticity of the acid-denatured ferricytochrome *c* (Fig. 2a), indicating that the high concentrations of crowding agent induce the secondary structure in the acid-denatured ferricytochrome *c*. Fig. 2b shows the effect of concentration and size of crowding agent on the 222 nm ellipticity of acid-denatured ferricytochrome *c* at pH 2.0. Clearly, among the dextran 40 and dextran 70, the negative cotton effect at 222 nm for acid denatured ferricytochrome *c* is more pronounced in the presence of dextran 40 than in the presence of dextran 70. These findings indicate that the smaller size crowding agents are more effective in increasing the secondary structure of the acid-denatured ferricytochrome *c* than that of the larger size crowding agents. Fig 2c shows that the variation of 222 nm ellipticity of acid-denatured

ferricytochrome *c* with [Na_2SO_4] at pH 2.0, which clearly shows that the acid denatured ferricytochrome *c*, acquires substantial secondary structure in the presence of salt.

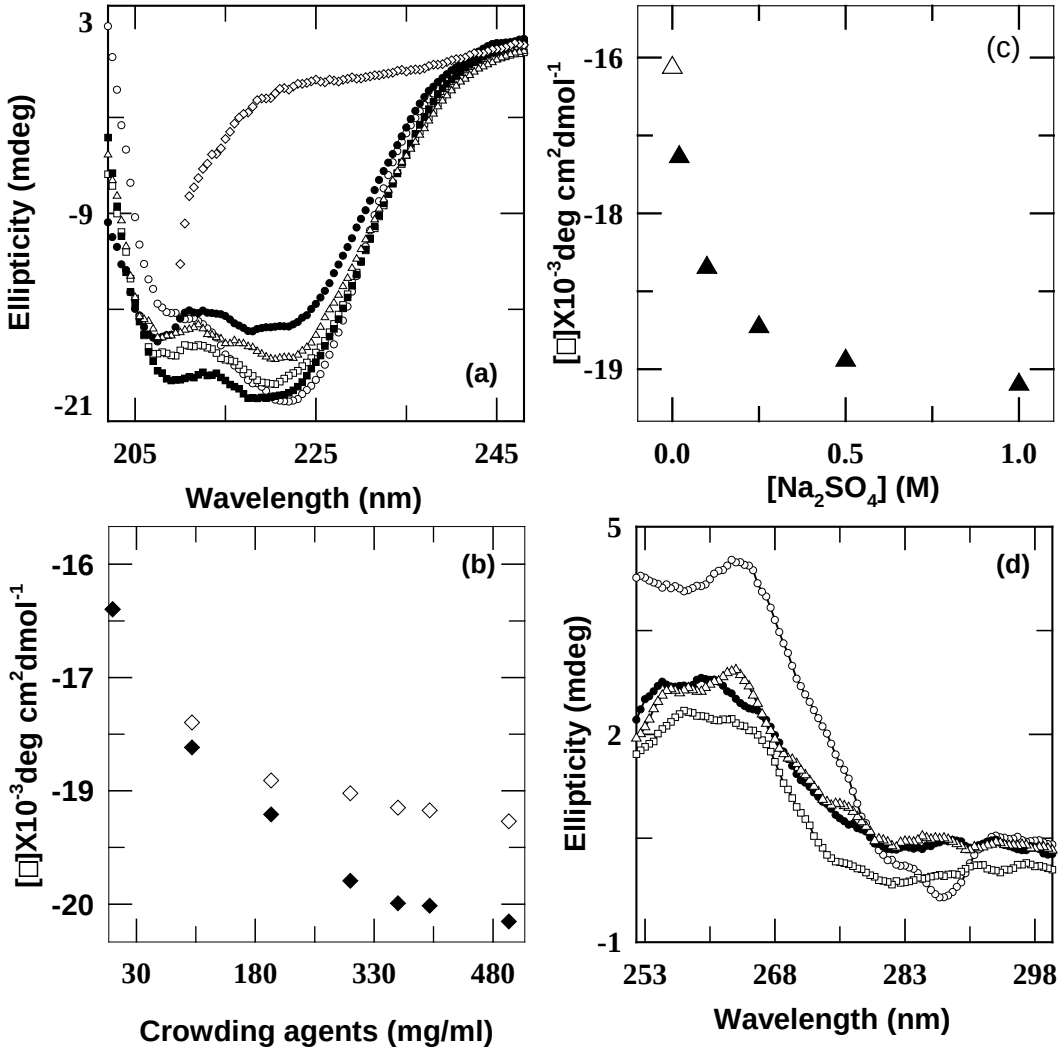

Figure 2. Panel (a) presents far-UV CD spectra for different states of ferricytochrome *c*: pH 7.0, native state (○); GdnHCl (5.0 M) unfolded state (pH 7.0) (◊); acid-denatured state of ferricytochrome *c* (pH 2.0) (•); 300mg/ml dextran 70 induced MG-state of acid-denatured ferricytochrome *c* (Δ); 300mg/ml dextran 40 induced MG-state of acid-denatured ferricytochrome *c* (■), and 1.0 M Na_2SO_4 induced MG-state of acid-denatured ferricytochrome *c* (□) (b) Peptide ellipticity of acid-denatured ferricytochrome *c* (pH 2.0) as a function of dextran 70 (◊) and dextran 40 (♦) (c) Peptide ellipticity of acid-denatured ferricytochrome *c* as a function of Na_2SO_4 (▲). (d) Near-UVCD spectra for different state of ferricytochrome *c*: native state (○); acid-denatured state of ferricytochrome *c* (pH 2.0) (•); 400mg/ml dextran 70 induced MG-state of acid-denatured ferricytochrome *c* (Δ); and 1.0 M Na_2SO_4 induced MG-state of acid-denatured ferricytochrome *c* (□)

3.3 Acid-denatured ferricytochrome *c* not acquire tertiary structure in the presence of crowding agents

Fig 2(d) shows the near-UV CD spectra for the different states of ferricytochrome *c*. The native state of ferricytochrome *c* at pH 7.0 exhibits two negative peaks at 282 nm and 289 nm (open circle line) spectrum, Fig. 2d) that arise from the tyrosyl side chains [40-41]**.** The near–UV CD absorption band for ferricytochrome c (282 and 289 nm) are substantially disrupted at pH 2.0 (up triangle line), indicating a significantly loss of tertiary structure of the pH-denatured protein. The acid-denatured protein not regain the near-CD absorption band at 282 and 289 nm in the presence of ~400 mg/ml dextran 70 (Δ) or 1.0 M Na_2SO_4 (□), indicating that the acid-denatured ferricytochrome *c* not acquire the tertiary structure in the presence of crowding agents or salt.

3.4 Effect of crowding agents on the GdnHCl-induced equilibrium unfolding of acid-denatured ferricytochrome *c*

Fig. 3 shows the fluorescence-monitored GdnHCl-induced equilibrium unfolding curves of acid-denatured ferricytochrome *c* (pH 2.0) collected in the absence (open circle) and presence of 300 mg/ml dextran 40 (filled circle) or dextran 70 (filled squire) at 25 ºC. The inclusion of crowding agent results in the shift of the unfolding curves towards higher GdnHCl concentrations, indicating that the crowding agents increase the stability of acid-denatured ferricytochrome *c*. Clearly, among the dextran 40 and dextran 70, the smaller size dextran 40 shifts the GdnHCl unfolding curve of acid-denatured ferricytochrome *c* more toward higher GdnHCl concentrations than that of the larger size dextran 70 (Fig. 3). This finding indicates that smaller size crowding

agent is more effective in increasing the stability of the acid-denatured ferricytochrome *c* than that of the larger size crowding agents.

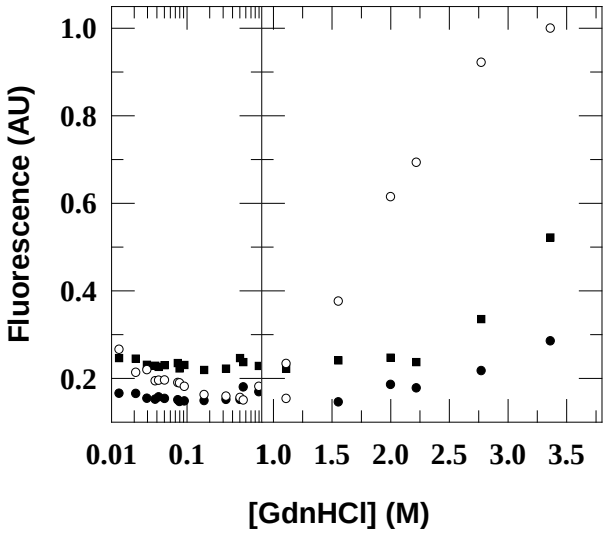

Fig 3:- The GdnHCl-induced folding unfolding transition of acid-denatured ferricytochrome *c* at pH 2 in the absence (○) and presence of ~300mg/ml dextran 40 (●) or dextran 70 (■).

4.0 Discussion

The present work discusses the effects of concentration and size of crowding agent on structure, folding and stability of acid-denatured ferricytochrome *c* at pH 2.0. The crowding agents stabilize and refold the acid-denatured ferricytochrome *c* (pH 2.0) to MG-states. Interestingly, the stabilization of acid-denatured ferricytochrome *c* to MG-state is more favorable in the presence of smaller size crowding agent (dextran 40) than that of the larger size crowding agent (dextran 70).

4.1 Macromolecular crowding effects transform acid-denatured ferricytochrome *c* to MG-state.

On decreasing the pH from 7 to 2.0 the protein becomes positively charged. The resulting intramolecular repulsion between the positively charged groups leads to acid-unfolding of the protein. The result obtained indicates that the crowding agents have a great impact on molecular compaction of acid-denatured ferricytochrome *c*. It is observed that the fluorescence emission intensity of the acid denatured ferricytochrome *c* decrease significantly in the presence of crowding agent (Fig. 1b), which is found to be more decrease for the smaller size crowding agent (dextran 40) than that of the larger size crowding agent (dextran 70), indicating that the molecular crowding effects cause molecular compaction of acid-denatured ferricytochrome *c*. Furthermore, the molecular compaction of acid-denatured ferricytochrome *c* by ~300 mg/ml dextran 40 is comparable that of the 1.0 M Na_2SO_4 (Fig. 1b), an essential property of MG-state.

At pH 2.0, the acid-denatured ferricytochrome *c* substantially loss its secondary structure element. However, in the presence of high concentrations of crowding agent, the acid-denatured ferricytochrome *c* regains substantial content of secondary structure. Furthermore, the secondary structure of acid-denatured ferricytochrome *c* in the presence of ~400 mg/ml dextran 40 is comparable with the secondary structure of native protein and acid-denatured ferricytochrome *c* in the presence of ~1.0 M Na_2SO_4, indicating that macromolecular crowding effect induce the native like secondary structure in the acid-denatured ferricytochrome *c*, which is an essential property of MG-state. Interestingly, the acid-denatured ferricytochrome *c* not acquire the tertiary interactions in the presence of high concentrations of crowding agents, confirming that the macromolecular crowding effects stabilize and refold the acid-denatured ferricytochrome *c* to MG-state.

5.0 Conclusion

The present work examines the effect of concentration and size of crowding agents (dextran 40 and dextran 70) on structure, stability and folding of acid-denatured ferricytochrome c at pH 2.0. It has been found that the high concentrations of crowding agent cause molecular compaction of acid-denatured ferricytochrome *c*. The crowding induced molecular compaction of acid-denatured ferricytochrome *c* is more pronounced for the smaller size crowding agent (dextran 40) than that of the larger size dextran 70. The acid-denatured ferricytochrome *c* also gains substantial secondary structure content in the presence of high concentrations of crowding agent. The crowding-induced secondary structure content is also found more for the smaller size crowding agent (dextran 40) than that of the larger size dextran 70. Remarkably, the acid-denatured ferricytochrome *c* not acquire tertiary interaction in the presence of high concentrations of crowding agents, indicating that the molecular crowding effects transform the acid-denatured ferricytochrome *c* to MG-state.

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