Biophysical Chemistry 163–164 (2012) 56–63

Contents lists available at SciVerse ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

# Ion specific influences on the stability and unfolding transitions of a naturally aggregating protein; RecA☆

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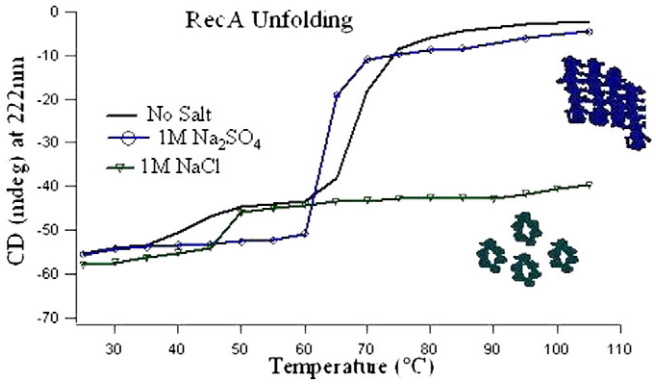
## HIGHLIGHTS

- Chloride salts lead to altered RecA complexes resistant to complete denaturation.
- Anion influences follow the reverse Hofmeister series.
- Changes in RecA stability do not correlate with differences in CD spectra.
- Changes in RecA stability correlate with changes in aggregation states.
- Salt effects result from a complex set of ion–protein and ion–solvent interactions.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 9 January 2012
Received in revised form 13 February 2012
Accepted 18 February 2012
Available online 24 February 2012

*Keywords:*
Hofmeister series
Salt
RecA
Circular dichroism
Aggregation
Unfolding

## ABSTRACT

The *Escherichia coli* RecA protein is a naturally aggregated protein complex that is affected by the presence of salts. In order to gain further insight into the nature of the ion-interactions on a naturally aggregating protein we used circular dichroism (CD), fluorescence and dynamic light scattering (DLS) to study the effects of different concentrations of $MgCl_2$, $CaCl_2$, NaCl, $Na_2SO_4$, and $MgSO_4$ on RecA structure and thermal unfolding. The results show unique ion influences on RecA structure, aggregation, unfolding transitions and stability and the anion effects correlate with the reverse Hofmeister series. The mechanisms of the ion-induced changes most likely result from specific ion binding, changes in the interfacial tension and altered protein–solvent interactions that may be especially important for protein–protein interactions in naturally aggregating proteins. The presence of some ions leads to the formation of RecA complexes that are resistant to complete denaturation and nonspecific aggregation.

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*Abbreviations:* CD, Circular Dichroism; Tris, tris(hydroxymethyl)aminoethane; MANT, 2′-3′O-(N-methylanthraniloyl); DLS, Dynamic Light Scattering.

☆ This research was supported by NSF RUI-0814716, NSF-REU 0754521, NSF MRI CHE-0420877. This work was performed, in part, at the Center for Integrated Nanotechnologies, a U.S. Department of Energy, Office of Basic Energy Sciences user facility at Los Alamos National Laboratory (Contract DE-AC52-06NA25396) and Sandia National Laboratories (Contract DE-AC04-94AL85000).

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doi:10.1016/j.bpc.2012.02.005

## 1. Introduction

Many studies on model systems and proteins have shown that ions in the Hofmeister series interact with macromolecules and water to alter protein aggregation and stability and are reviewed recently [1–3]. Initially Hofmeister proposed a series of anions $CO_3^{2-} > SO_4^{2-} > H_2PO_4^{2-} > F^- > Cl^- > NO_3^- > I^- > ClO_4^-$ and a series of cations: $K^+ > Na^+ > Mg^{2+} > Ca^{2+}$. The kosmotropic ions on the left tend to precipitate proteins while the chaotropes on the right tend to enhance protein solubility and favor denaturation [4]. There has been an enormous amount of research studying the ion's influence on model polymers, protein and water structure. Studies have now shown that salts can directly interact with proteins and may influence interactions by Debye–Hückel screening of charges, changing hydration at water interfaces and/or altering water structure and changing the hydration of the protein [2,5–7]. Numerous studies have shown how the different ions influence protein folding and aggregation and highlight the importance of the ions influence on solution surface tension, dispersion forces, electrostatic screening, ion-hydration, and interactions both with the peptide backbone and specific charged groups on the protein or peptide, for examples see [5,8–15]. Molecular dynamics simulations also support specific ion–protein interactions with both charged or polar parts of the protein as well as hydrophobic regions of the protein [16–18]. Interactions between ions, water and proteins are dependent on concentrations and ion and protein properties, for some recent examples see [19–24].

Here we show that RecA, an aggregated protein complex shows similar behavior in that it tends to follow the reversed anionic Hofmeister series. In general, the mechanisms of salt-induced changes in protein aggregation are of particular interest for their role in the prevention or facilitation of toxic aggregated, fibril formation in prion proteins [25,26] and in amyloidogenic peptides and proteins [27,28]. However, fewer studies have been performed that investigate the influence of salts on proteins that are aggregated in their normal, functioning complexes. Because salt ions have distinct influences on the RecA protein and it normally exists as an aggregated complex it provides an interesting system for studies of how different ions alter the structure, stability and unfolding of an aggregated protein.

RecA, an *Escherichia coli* protein, performs DNA repair, genetic recombination, can initiate the SOS response and is reviewed in [29]. Comparison of the RecA structures and electron microscopy studies show protein filaments with differences in the pitch of the protein helix and rotations of the C-terminal domain (for some examples see [30–33]). RecA exists as mixtures of small particles, oligomers, rods, larger aggregated filaments and bundles whose distribution is dependent on protein concentration [34,35]. Addition of NaCl, $CaCl_2$ or $MgCl_2$ alters the distribution in a concentration and ion dependent manner [34–36] and the addition of $CuCl_2$, $ZnCl_2$, or $HgCl_2$ inactivate and precipitate RecA [37]. Salts also have distinct effects on RecA activity, and stability [38,39]. Understanding the influence of salt ions on RecA stability may be of interest to those scientists who have shown new and interesting applications of RecA such as using porous vesicles to modulate RecA–DNA interactions and synthesizing conductive metal nanowires [40,41].

In the present study we have investigated the influence of a variety of salts on the structure and the thermal denaturation profiles of RecA. CD was used to monitor the unfolding of RecA in various concentrations of different salts that included some conditions known to activate the ATPase activity in the absence of DNA [38]. Turbidity measurements and dynamic light scattering studies confirm that salts differentially alter RecA aggregation. Fluorescence experiments showed that nucleotide binding can occur in the presence of high concentrations of sodium chloride. We conclude that altering the ionic environment of RecA results in complex differences in stability and structural intermediates that are concentration dependent and more consistent with protein systems that follow the reversed anionic Hofmeister series. The present investigation provides evidence that ion–protein and ion–water interactions modulate RecA structure, solvent exposed surface area, aggregation state, stability and the thermally induced unfolding transitions. In addition, we have identified some solution conditions that allow RecA to form thermally stable aggregates that may be of interest to scientists working to create novel protein nanostructures and to control protein non specific aggregation processes.

## 2. Materials and methods

### 2.1. Preparation of RecA

*Escherichia coli* RecA was purchased from New England Biolabs (Ipswich, MA) and exchanged into storage buffer (20 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM $MgCl_2$) using an Ultrafree centrifugal filter device (10 KD Biomax membrane). Protein was stored at −20 °C until use.

### 2.2. Circular dichroism studies of thermal unfolding

RecA was diluted into the previously described Tris buffer or into the Tris buffer with the addition of one of the following salts: NaCl, $MgCl_2$, $CaCl_2$, $Na_2SO_4$, or $MgSO_4$. All CD experiments were performed with a final RecA concentration of 5 μM. For some experiments additional cofactors were included such as 7.5 μM MANT-ADP (Invitrogen Corp). CD and fluorescence experiments were performed using a 3 mm path length Spectrosil® Far UV quartz cell from Starna Cells, Inc (Atascadero, CA) and a Jasco J-810 spectropolarimeter with a single Peltier cell holder with fluorescence capability (PFD-425) for temperature control. Signal to noise was enhanced by increasing the nitrogen purge flow rate to 100 L/min for all experiments. Three accumulations were co-added to obtain each CD spectrum presented. CD spectra of buffer alone were subtracted from RecA spectra using Jasco Spectra Analysis software. CD spectra were obtained in buffer solutions and protein samples at 25 °C from 300 nm to 180 nm in continuous mode (scanning speed, 20 nm/min; data pitch, 0.1 nm; bandwidth, 1 nm; response time, 4 s). CD data were obtained from 25 °C to 105 °C in 5 °C intervals with 2 minute equilibrations at each temperature prior to data collection. Data for thermal melting profiles were collected with a 100 nm/min scanning speed with other parameters remaining unchanged. In attempts to obtain high signal-to-noise data and follow small structural transitions, higher concentrations of RecA were used such that the 208 nm HT voltages were increased in some samples, especially around the melting temperatures of the protein. The value of the HT voltage is larger when more light is absorbed or scattered, but in all cases the 216 nm and 222 nm voltages were within accepted limits (<700 V at 216 nm and significantly lower for 222 nm). Therefore, the thermally induced loss of secondary structure was monitored by plotting ellipticity at 222 nm as a function of temperature. These plots were used to monitor transitions and obtain melting temperatures under all conditions. Protein aggregation was monitored on the identical samples by plotting the voltage (HT) signal at 285 nm as a function of temperature which provides a measure of turbidity of the sample [42].

### 2.3. MANT-ADP fluorescence studies

Direct fluorescence and FRET studies were performed in the presence of MANT-ADP. All solutions contained 5 μM RecA and 7.5 μM MANT-ADP. Fluorescence was monitored in step scan mode at $\lambda_{em}$ 280–500 nm (data pitch, 1 nm; bandwidth, 10 nm, response time, 1 s; slit width, 350 μm). Fluorescent resonance energy transfer was monitored by excitation of RecA tryptophan and tyrosine residues at an $\lambda_{ex}$ 270 nm while direct excitation of the MANT nucleotide was achieved at an $\lambda_{ex}$ 356 nm. Data were collected at 25 °C following a

2 minute equilibration. Unfolding data were obtained in 10 °C intervals between 35 °C and 85 °C by allowing samples to equilibrate for 2 min at temperature, lowering the temperature to 25 °C for 2 min and collecting data. CD data were collected simultaneously to ensure repeated heating and cooling of the samples did not lead to significant changes in secondary structure when compared to data collected as previously described.

### 2.4. Dynamic light scattering studies of thermal unfolding

RecA was diluted to 5 μM in 20 mM Tris–HCl pH 7.5 or into the same buffer with the addition of one of the following salts (final concentration): NaCl (250 mM, 1 M, or 2 M), $MgCl_2$ (1 M), $CaCl_2$ (1 M), $Na_2SO_4$ (1 M), or $MgSO_4$ (1 M). All buffer, salt solutions, and protein were filtered through a 0.45 μm filter (SpinX; Costar, Corning Inc) prior to mixing. Diluted protein was equilibrated with buffer and salts for 25 min at room temperature. DLS experiments were performed using a small volume quartz cuvette (QS 3.00 mm cell) (Starna Cells) on a Zetasizer NanoZS (Malvern Instruments). Temperature was varied from 25 °C to 85 °C, with 10 °C intervals. Control experiments were completed with all buffer and salt combinations, without protein and monitored as a function of temperature. All experiments were repeated a minimum of two times and were consistent between RecA preparations. All temperature and salt conditions demonstrated reproducible correlation functions.

## 3. Results

### 3.1. Circular dichroism studies of salt influences on RecA Structure

Fig. 1 shows CD spectra obtained of RecA in different solution conditions. Fig. 1a shows an overlay of the spectra obtained at 25 °C in 1 M concentrations each of the salt solutions studied. The control RecA spectrum is generally similar to the spectra obtained in the presence of 1 M of $MgSO_4$, $Na_2SO_4$, and NaCl. However, RecA in the presence of $MgCl_2$ and $CaCl_2$ shows additional signal near 208 nm that may suggest that these salts stabilize α-helical structure (Fig. 1a). Sodium chloride and sulfate salts (Fig. 1a) do not show a similar increase.

In order to investigate any concentration dependent changes in structure we compared the control (no salt) spectrum and RecA spectra obtained in the presence of 250 mM, 1 M and 2 M NaCl (Fig. 1b). Comparison of spectra obtained in different concentrations of $Na_2SO_4$ reveals that RecA spectra in 250 mM and 1 M $Na_2SO_4$ are similar to the control while the 2 M $Na_2SO_4$ spectrum is the only condition that resulted in large, overall changes reflecting altered RecA structure (Fig. 1c and Supplemental material). It should be noted that the 2 M $Na_2SO_4$ samples had the highest HT voltages suggesting that the altered structure is associated with increased aggregation at lower temperatures. For all of the salts and concentrations presented in this paper, 2 M sodium sulfate is the only salt that resulted in significant changes in shape of the RecA CD spectrum (see Supplemental material).

### 3.2. Circular dichroism studies of RecA thermal unfolding

The CD studies of thermally induced unfolding of RecA in the presence of 1 M salt concentrations are summarized in Fig. 2. 250 mM and 2 M concentrations of each salt were also studied and the results are shown in the Supplemental material. RecA in 1 M sodium chloride showed the greatest amount of retained signal at higher temperatures (Fig. 2b) and does not lose as much overall structure as the magnesium and calcium salts (Fig. 2b, c, d). Magnesium and calcium chloride (Fig. 2c, d) have similar melting profiles with more significant initial loss of intensity at 210 nm as compared to sodium chloride samples. RecA in 1 M concentrations of all tested chloride salts shows a significant amount of the signal around 216 nm at the highest temperatures suggesting a stabilized β-structure (Fig. 2b, c, d). In contrast, RecA in 1 M sulfate salts retains more overall structure at the lower temperatures, lacks the low temperature transition and has lower melting temperatures than the control samples (Fig. 3e, f). The CD signal at 210 nm for RecA in some of the 1 M chloride samples was unreliable above the transition temperatures for some of the salts (HT voltage was greater than 700 V) and the 216 and 222 nm HT voltages were always below accepted values, therefore we used the intensity of the CD signal at 222 nm to obtain melting profiles for RecA unfolding.

### 3.3. Thermal unfolding curves from circular dichroism and dynamic light scattering studies

Plotting the intensity of the CD signal at 222 nm as a function of temperature (Fig. 3a, b, c) allows us to easily obtain melting temperatures. For the control and many of the chloride samples we observe two transitions for RecA in the absence of salt, with the smaller transition at 45 °C and the larger transition at 65 °C (Figs. 2 and 3). The larger transition at 65 °C is associated with the complete unfolding

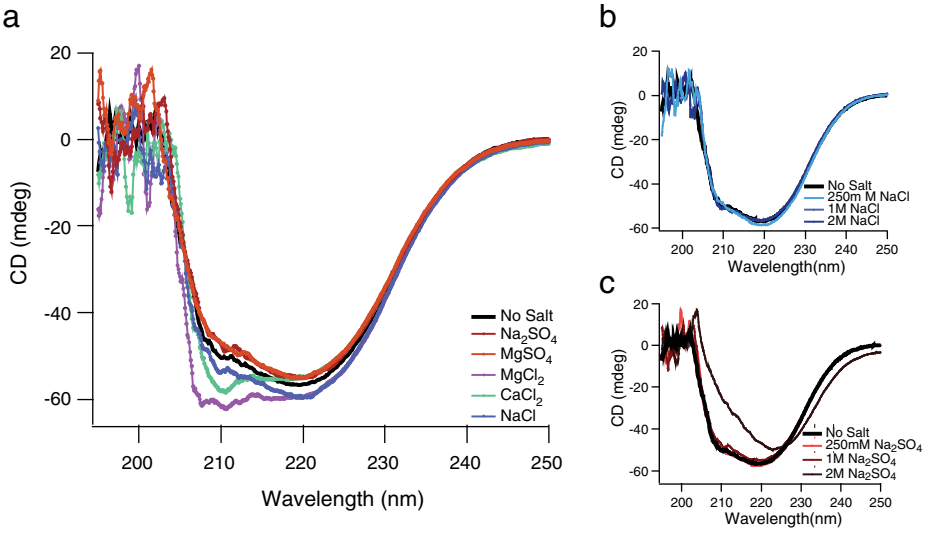


**Fig. 1.** Circular dichroism spectra of RecA at 25 °C. All solutions contained 5 μM RecA in Tris buffer (pH 7.5). (a) CD spectra of RecA in Tris buffer control (no salt) and 1 M concentrations of each of the salts $MgCl_2$, $CaCl_2$, $MgSO_4$, $Na_2SO_4$, NaCl. (b) CD spectra of RecA no salt and with 250 mM, 1 M or 2 M NaCl. (c) CD spectra of RecA with no salt and with 250 mM, 1 M or 2 M $NaSO_4$.

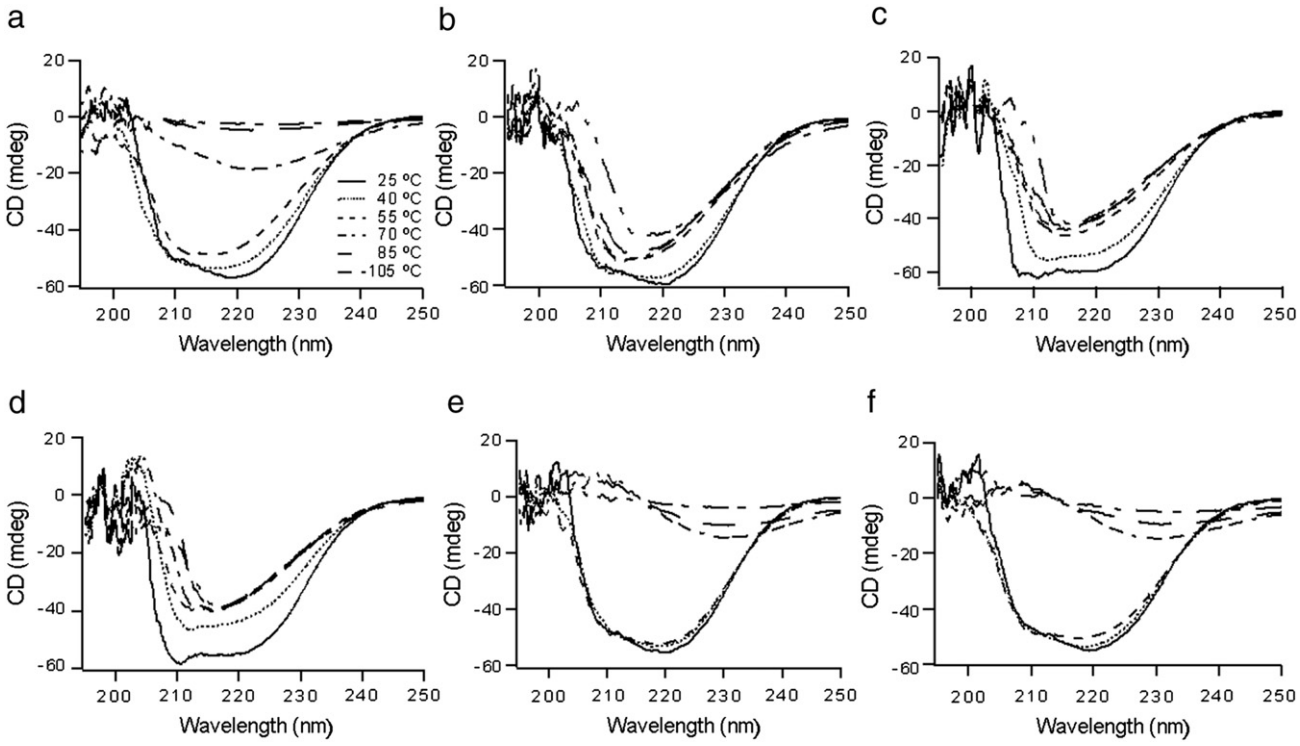


**Fig. 2.** Circular dichroism spectra of 5 μM RecA unfolding (25–105 °C) in 1 M salts. All solutions contained 5 μM RecA in Tris buffer (pH 7.5) (a) with no salt and the addition of 1 M concentrations of (b) NaCl; (c) $MgCl_2$, (d) $CaCl_2$; (e) $Na_2SO_4$; and (f) $MgSO_4$. For each panel spectra obtained at 25, 40, 55, 70, 85 and 105 °C are shown.

of RecA (Fig. 3a–c). At 1 M concentrations RecA unfolds similarly depending on the anion present (Figs. 2 and 3b). The chloride salts (different total ionic strengths) show initial loss of structure at the lower temperatures, a CD transition at 45 °C and retention of 222 nm signal even at 105 °C (~73%). Some differences are observed such as the greater loss of signal in calcium chloride. The turbidity (HT voltage at 285 nm) measurements were obtained on the CD samples to simultaneously assess changes in RecA aggregation state [42]. The 1 M

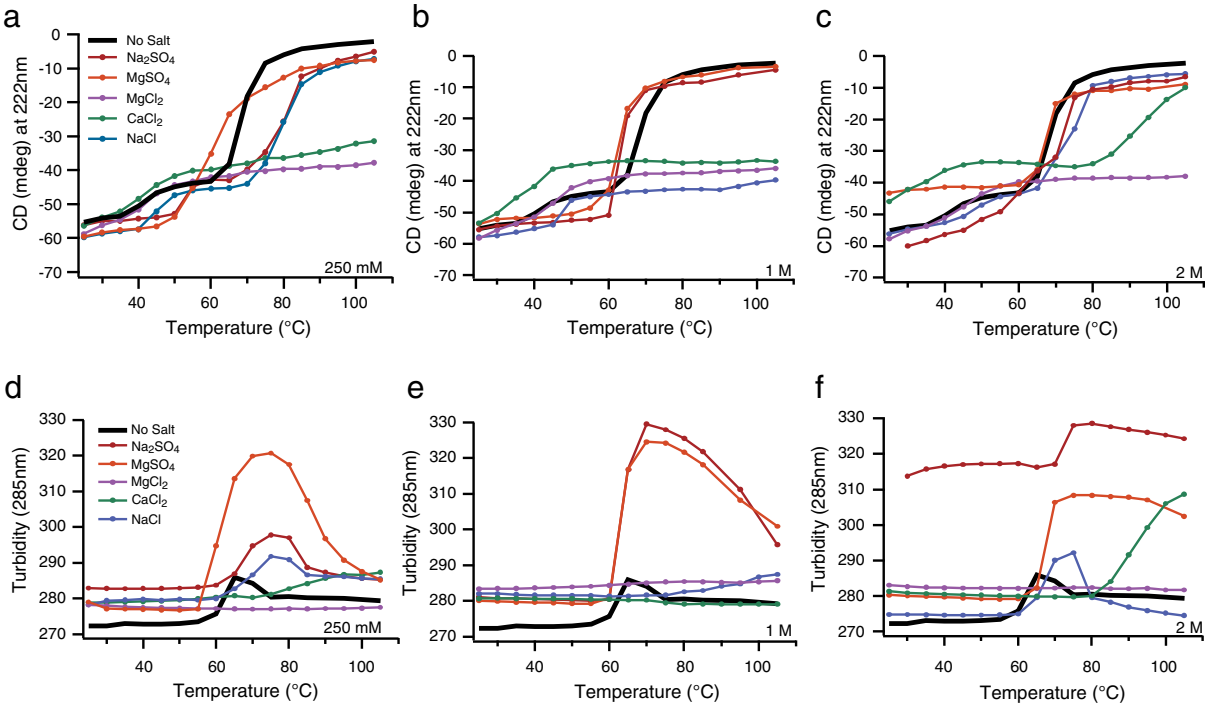


**Fig. 3.** Plots of CD intensities and HT voltages as a function of temperature. All solutions contained 5 μM RecA in Tris buffer (pH 7.5) and addition of 250 mM, 1 M or 2 M of the following salts: $MgCl_2$, $CaCl_2$, $MgSO_4$, $Na_2SO_4$ or NaCl. The intensity of the CD signal at 222 nm was plotted against temperature for RecA in (a) 250 mM salts (b) 1 M salts (c) 2 M salts. HT voltage at 285 nm obtained on the identical samples used to generate CD spectra in A,B and C plotted as a function of temperature for RecA in (d) 250 mM salts, (e) 1 M salts and (f) 2 M salts.

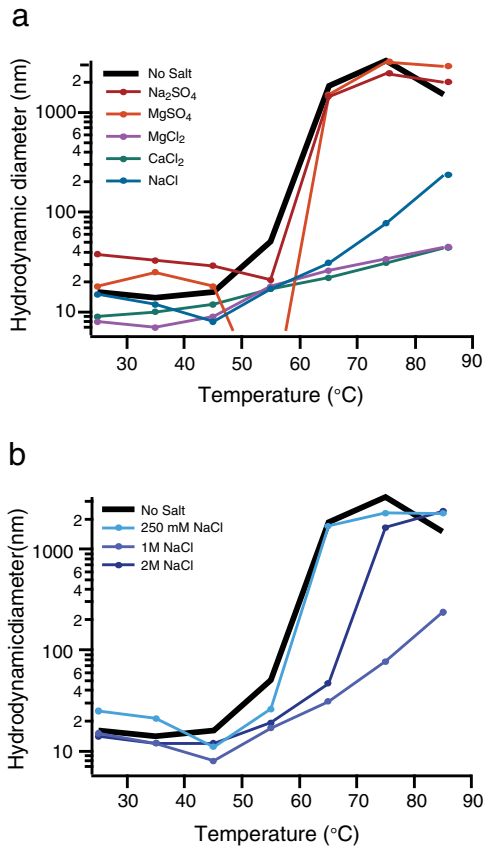


**Fig. 4.** Trends in protein sizes as a function of temperature (dynamic light scattering) in (a) Tris buffer with 1 M $MgCl_2$, $CaCl_2$, $MgSO_4$, $Na_2SO_4$ or NaCl and (b) 250 mM, 1 M and 2 M NaCl.

chloride salts showed no significant change as a function of temperature (Fig. 3e). The HT voltage plots and DLS data (Fig. 4a) show large increases around 60 °C for RecA in sulfate salts that correlate with the reduction in CD intensity (Fig. 2e, f) and are consistent with non-specific aggregation. The percent average error for the DLS experiments was: Tris (4%); 250 mM NaCl (7%); 1 M NaCl (13%); 2 M NaCl (20%); 1 M MgCl2 (7%); 1 M CaCl (15%); 1 M MgSO4 (14%); and 1 M Na2SO4 (10%). DLS and turbidity measurements correlate and provide additional evidence that turbidity measurements can provide information about the aggregation state [42].

### 3.4. Fluorescence studies of nucleotide binding

Fluorescently labeled nucleotides were used to study how salt solutions influence nucleotide binding to RecA. Fig. 5 shows results obtained by performing modified unfolding experiments in the presence of the MANT-ADP. In order to ensure that temperature was not influencing the fluorescence spectra of the MANT-nucleotides, RecA samples incubated at higher temperatures were then cooled to 25 °C for fluorescence experiments. CD spectra of each sample were obtained concurrently to ensure that these spectra were similar to those used for the thermal unfolding curves. Fig. 5 (b, c) shows the MANT-nucleotide alone (solid line) and shows how the fluorescence of the nucleotide is increased in the presence of RecA (no salt) at 25 °C (Fig. 5b) as would be expected if the MANT nucleotide is able to bind to the protein. Fig. 5b also shows increased fluorescence, thus nucleotide binding at 35 °C and 45 °C but not at 55 °C. Fig. 5c shows that exciting the protein (no salt) results in significant energy transfer to the MANT nucleotide at 25 °C, 35 °C and 45 °C but not at 55 °C. Excitation of the MANT-ADP (Fig. 5e, h) shows that data from NaCl RecA samples have increased fluorescence for 25, 35, 45 and 55 °C but only show increases for 25, 35, and 45 °C if RecA is excited (Fig. 5f, i).

## 4. Discussion

Our CD results show small changes in RecA structure in the presence of magnesium and calcium chloride (Fig. 1a). Low concentrations of $Mg^{2+}$, cause the RecA C-terminus to transition to a more open conformation [43]. N-terminally truncated RecA shows changes in α-helical structure alter protein–protein interactions and theoretical studies of RecA proteins indicated that α-helices play a key role in thermal stability [44,45]. Our CD spectra are nearly identical in the presence of different concentrations of sodium chloride. Previous studies with lower RecA concentrations in the presence of ATP showed an increase in α-helical structure with the addition of NaCl [46]. Our results agree with other previous studies that showed no significant change in structure associated with high concentrations of NaCl [47].

CD studies provide interesting insights about how different ions and concentrations of ions alter RecA structure, aggregation and unfolding intermediates. Our combined results may be explained by the different anions stabilizing different initial aggregates and suggest that partial denaturation and/or a change in aggregation state could be reflected in the initial transition around 45 °C that is observed for RecA in the presence of chloride. Comparison of the DLS data (Fig. 4a and b) and the turbidity measurements (Fig. 3e) suggest that at 1 M concentrations and at higher temperatures the chloride stabilizes smaller, more thermostable aggregates than the control. Previous studies on *Thermus thermophilus* RecA (ttRecA) in 1 M KCl and urea showed similar changes and two transitions that the authors ascribed to the dissociation of the hexamer and then monomer unfolding [39]. In contrast, our DLS data show increases in light scattering at 55 °C, and suggest small, temperature dependent increases in aggregation in the absence of urea and the presence of 1 M NaCl.

In contrast to chloride, 1 M concentrations of sulfates stabilize structures then unfold at lower temperatures suggesting a reverse anionic Hofmeister series that could arise from RecA surface properties [19]. Studies performed at additional salt concentrations reveal concentration dependent influences on stability and intermediate structures. Stabilization is not observed at 125 mM $MgCl_2$ (Supplemental Fig. 3) while RecA in 2 M $MgCl_2$ retains the majority of the CD signal even at 105 °C. This stabilization is not due simply to ionic strength as the calcium chloride samples unfold at higher temperatures. 2 M $Na_2SO_4$ samples show that increased aggregation (Fig. 3f) may result from the altered structures observed in the CD spectrum (Fig. 1c). The 2 M and 250 mM studies (Fig. 3a, d) reinforce a differential influence of the cations on the thermal stability ($Na^{+} > Mg^{2+}$) and correlate with what is predicted from the cationic Hofmeister series for the sulfate salts [4]. This correlation is not observed for the chloride salts, in fact at 2 M concentrations of chloride salts we again see the reverse Hofmeister series for the cations studied.

The CD and DLS data clearly show differences in structural transitions and aggregation states are observed in the presence of the different ions. Complementary information was also obtained by studying nucleotide binding to RecA in the various salt conditions. These experiments were performed in attempts to indirectly probe the structure of the nucleotide binding site. The results presented in Fig. 5 suggest that 1 and 2 M NaCl RecA samples bind nucleotide but bind less MANT-ADP as compared to the control samples at 25–45 °C (compare Figures B with E and H). There was no significant increase in fluorescence or suggestion of nucleotide binding observed for RecA-MANT-ADP samples in the other 1 M salt solutions (data not shown). The unique NaCl aggregates are able to bind the MANT-ADP at lower temperatures.

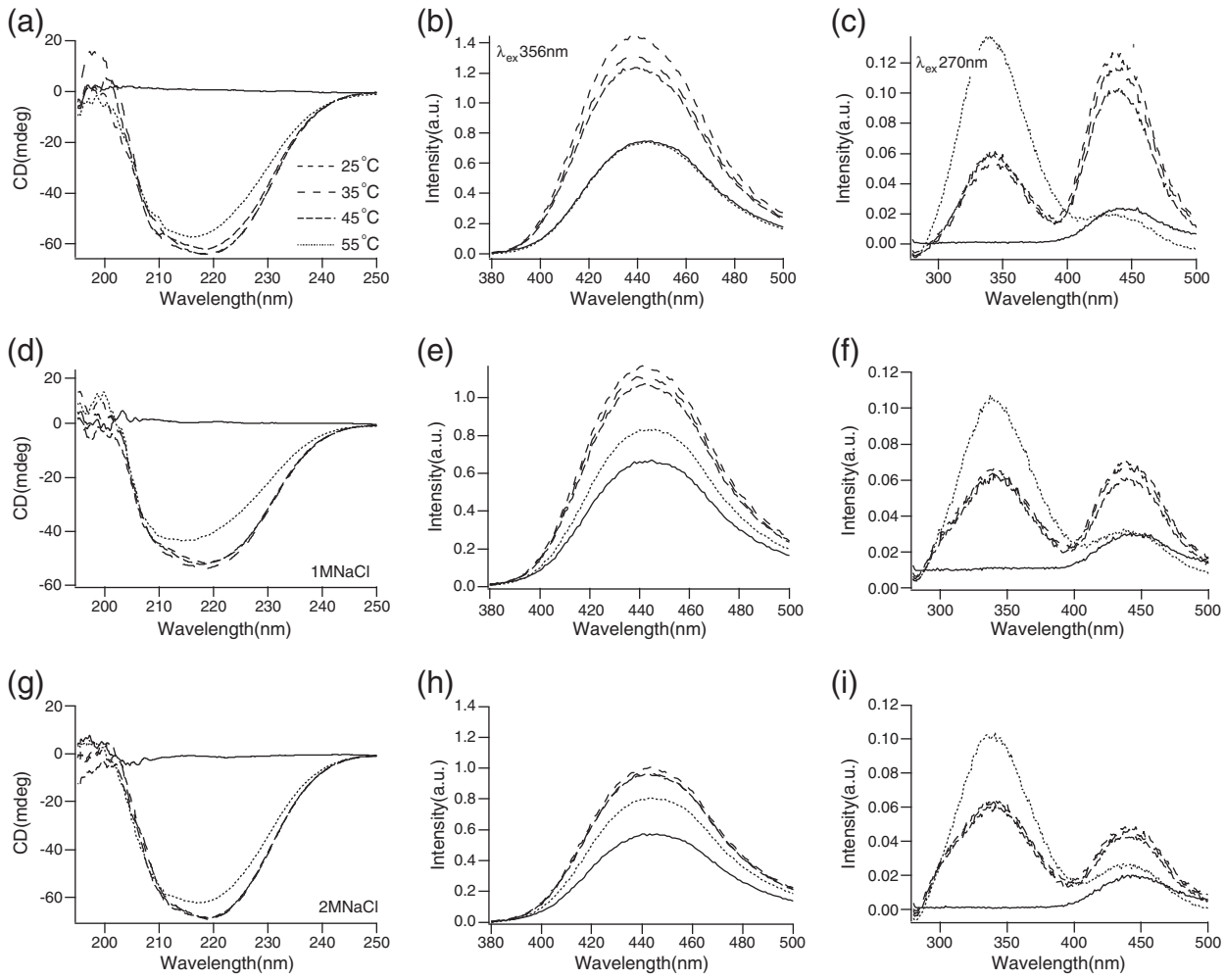


**Fig. 5.** Fluorescence studies of MANT-ADP binding to RecA. RecA interaction with the MANT-ADP nucleotide analog monitored by Far-UV CD, direct fluorescence, and FRET at 25, 35, 45, and 55 °C. MANT-ADP was measured at 25 °C (——). Spectra were obtained in the absence (a–c) and presence of 1 M and 2 M sodium chloride (d–f and g–i). Direct fluorescence data (b, e, h) was obtained by excitation of the MANT-ADP nucleotide ($\lambda_{ex}$ 356 nm) and FRET data (c, f, i) was obtained by excitation of RecA tyrosine and tryptophan residues ($\lambda_{ex}$ 270 nm).

The experiments presented in this paper demonstrate that the solution and ionic environment has a significant influence on the aggregation and thermal unfolding of RecA. The solution conditions used here are much higher than those observed at more physiological concentrations of around 50 mM for $Cl^-$ and 150 mM $Na^+$ [48]. However, our very preliminary, unpublished data at lower concentrations around 5–50 mM show some similar trends for the anions but a larger dependence on the nature of the cation. The changes in stability at higher concentrations do not directly correlate with any significant change in the overall RecA structure. 1 M concentrations of all chloride salts stabilized RecA and had substantial amounts of structure retained at high temperatures. The DLS and turbidity measurements suggest anion dependent changes in aggregation state that are linked to enhanced thermal stability. The ability of anions to affect these transitions depends on a critical anion concentration somewhere between 250 mM and 500 mM (Supplemental Fig. 4) for NaCl RecA complexes and between 125 mM (Supplemental Fig. 3) and 250 mM for $MgCl_2$. RecA in 500 mM NaCl and 250 mM $MgCl_2$ have profiles similar to the 1 M concentrations. The influence of the cations on stability is most apparent at the 250 mM and 2 M concentrations.

This type of interplay between the cation and anion influences has also been observed in the human prion protein. Ronga et al. observed preferential binding of some anions and suggested the importance of cation effects [26]. In order to confirm an indirect correlation with the traditional anionic Hofmeister series we performed additional experiments on RecA in the presence of sodium perchlorate. Interestingly, the presence of 250 mM or 1 M $NaClO_4$ (Supplemental Fig. 5) also leads to intensity increases around 208 nm and RecA stabilization. Previous studies have shown that sodium perchlorate stabilizes helical peptides and that $ClO_4^-$ ions solvate the peptide backbone, and interact with amino groups and Arg side chains [14,15]. A similar result was found with β-lactoglobulin at pH 3 where $NaClO_4$ was more effective than NaCl or KCl in stabilizing the dimer and suggested a dominant role for the burial of hydrophobic surfaces [49]. Pugh and Cox also found an ion-specific nature of ATPase activation in the absence of DNA that correlates with our stability measurements as they observed increased activation by $Na^+$ as compared to $Mg^{2+}$ and increased activation by $Cl^-$ as compared to $SO_4^{2-}$ [38]. Computational studies have indicated that sulfate anions interact more strongly with protein and peptides than chloride ions and that sodium ions have only weak affinities for protein surfaces [16]. Studies have also shown that complex ion interactions between protein and counterion contribute to the ability to disrupt noncovalent interactions and alter protein stability [17]. For example, sodium salts increase the folding times of an oligopeptide by an order of magnitude [50]. The RecA studies provide additional evidence for a complex picture of ion induced influences on proteins.

The largest differences in RecA stability correlate with anion effects on aggregation and unfolding intermediates. Multiple explanations could account for the differences observed in our experiments. Ion–

water, ion–protein and ion induced changes in the interfacial tension could explain the differences we observe. Differences in ionic interactions have clearly shown that the RecA aggregation state is dependent on temperature and salt and protein concentrations, for examples see, [34,35]. Multiple crystal structures of compressed RecA filaments have provided insights about calcium, sulfate and phosphate binding to specific sites on the protein and how they influence intra and inter filament buried surface area [30]. At the higher concentrations of salts used in our studies it is likely that the ions bind to additional protein sites and influence the protein–water interfaces. The studies of ttRecA showed that the presence of 1 M KCl led to dissociation of the aggregate, formation of a hexamer and increased stability of RecA in the presence of urea [39]. Our experiments also suggest 1 M chloride ion stabilization of smaller aggregates correlates to increases in thermal stability.

Theoretical studies show that the reverse anionic Hofmeister series should be expected and the ion effects are dependent on the concentration of ions and the surfaces of the protein being studied [19]. Studies on positively charged lysozyme have shown that at lower (~200–300 mM) salt concentrations inverse Hofmeister effects are observed while at higher concentrations the anions correlate with Hofmeister predictions [22]. RecA, pI of ~5.6, [51] is negatively charged at pH 7.5, but also shows behavior that does not correlate with what is expected from the direct anionic Hofmeister series. Ion–water interactions also influence RecA–solvent and RecA–RecA interactions and could explain differences in protein structure and stability. Review of the effects of Hofmeister anions suggests a complex interplay of specific ion interactions with proteins, polymers and solvation water but suggest that the anions do not have large effects on the bulk water structure [1,2]. However, recent studies have shown that sulfate ions have longer range patterning effects on water [24]. In addition, some studies have also shown ion dependent specific interactions with amino acids, the peptide backbone and solvation water that can alter structures [13–18].

In comparison to these more recent studies, our results indicate that another level of complexity for ion–protein interactions may be associated with folded, aggregating proteins as solvent effects may become more pronounced in these self-aggregating systems. In particular, if we examine the case of the sodium chloride salts at 25 °C we see no concentration-dependent changes in the overall CD spectra, however, we do observe concentration dependent changes in RecA stability. Therefore, we must also consider the influence of ion–water interactions and interfacial tension. Studies have clearly shown that well hydrated and poorly hydrated anions affect phase transitions of polymers [52]. Previous studies showed that anion effects are dominated by the charge of the interfacial layer [7]. Experiments on myoglobin and bacteriorhodopsin show a relation between the interfacial tension and protein structural stability and reveal how there can be multiple mechanisms for ion induced changes in protein conformations [53]. These studies suggested fluctuations in protein structure are related to changes in interfacial tension [53]. Additional experiments have shown that protein function is coupled to motions in both the bulk solvent and the hydration-shell motions [54,55].

Overall, our results may be explained by the RecA surface area and ion-induced changes in interfacial tension and hydration that lead to alterations in the exposed surface area, protein flexibility, fluctuations and aggregation and result in our observation of some reverse Hofmeister effects. The RecA interactions will continue to change as denaturation alters the solvent exposed surface area. Molecular dynamics studies have shown that sulfate is strongly attached to the proteins but chloride ions do not interact strongly with the peptide [16]. This result combined with the effects observed in our paper suggests multiple mechanisms for the anions influencing protein stability and aggregation. The sulfate ions may pull water from the peptide and destabilize the protein by ion-binding, increasing interfacial tension and increasing the initial aggregation state resulting in an overall decrease in stability. However, the chloride ion-stabilization may have minimal chloride–protein interactions, increased protein hydration, smaller increases in interfacial tension and stabilize a smaller aggregate.

## 5. Summary

We have studied how different salts influence RecA structure, aggregation and thermal unfolding and have provided evidence that salt–RecA interactions result from specific ion binding events as well as ion-induced changes in the interfacial tension and protein–solvent interactions that alter the solvent-exposed surface area, aggregation and stability. The thermostability of residual RecA structure present in high concentrations of chloride solutions is not associated with a unique structure discernable by CD studies but more likely results from changes in protein–protein and protein–solvent interactions resulting in complexes that are resistant to complete denaturation and nonspecific aggregation. Some of these NaCl RecA samples show the ability to bind nucleotides at higher temperatures. We suspect that changes in interfacial tension and solvating waters may alter the exposed surface area of the protein and drive changes in aggregation suggesting that these changes are especially important for naturally aggregated proteins. Future experiments using additional spectroscopic probes could provide some interesting insights about the specific effects of the ions on RecA structure and RecA–solvent interactions. Additional nanoscale characterization of some of the stable protein complexes would aid in the development of novel, thermally stable, biological materials and lead to an increased understanding of solution conditions that could work against uncontrolled protein aggregation.

## Acknowledgments

This research was supported by NSF RUI-081476, NSF-REU 0754521, NSF-MRI and CHE-0420877. We would like to acknowledge NSF-REU participants Princess Bembong and Emanuel Lubert for their initial studies that led to the work presented in this paper. This work was performed, in part, at the Center for Integrated Nanotechnologies, a U.S. Department of Energy, Office of Basic Energy Sciences user facility at Los Alamos National Laboratory (Contract DE-AC52-06NA25396) and Sandia National Laboratories (Contract DE-AC04-94AL85000).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bpc.2012.02.005.

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