Biophysical Chemistry 184 (2013) 29–36

Contents lists available at ScienceDirect

Biophysical Chemistry

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

The effects of buffers and pH on the thermal stability, unfolding and substrate binding of RecA☆

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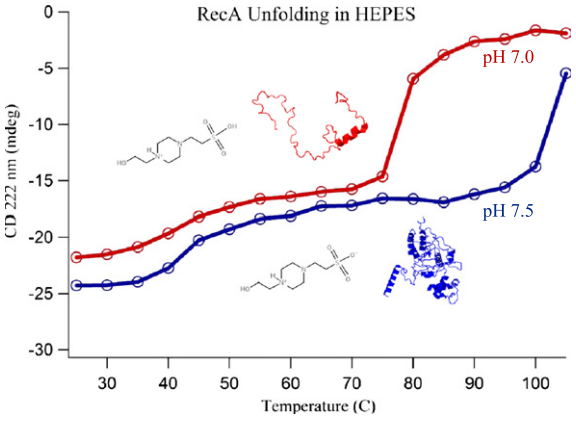
HIGHLIGHTS

- Increasing pH from 6.5 to 8.5 stabilizes RecA against thermal denaturation.
- Changes in RecA stability are not associated with changes in secondary structure.
- Buffering agents have large effects on RecA stability and dsDNA nucleation.
- Increased RecA stability is inversely correlated with DNA binding.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 July 2013
Received in revised form 6 August 2013
Accepted 6 August 2013
Available online 16 August 2013

Keywords:
Buffer effect
pH effect
RecA
Thermal unfolding
Circular dichroism
DNA binding

ABSTRACT

The *Escherichia coli* protein RecA is responsible for catalysis of the strand transfer reaction used in DNA repair and recombination. Previous studies in our lab have shown that high concentrations of salts stabilize RecA in a reverse-anionic Hofmeister series. Here we investigate how changes in pH and buffer alter the thermal unfolding and cofactor binding. RecA in 20 mM HEPES, MES, Tris and phosphate buffers was studied in the pH range from 6.5 to 8.5 using circular dichroism (CD), infrared (IR) and fluorescence spectroscopies. The results show all of the buffers studied stabilize RecA up to 50 °C above the Tris melting temperature and influence RecA's ability to nucleate on double-stranded DNA. Infrared and CD spectra of RecA in the different buffers do not show that secondary structural changes are associated with increased stability or decreased ability to nucleate on dsDNA. These results suggest the differences in stability arise from decreasing positive charge and/or buffer interactions.

Abbreviations: CD, circular dichroism; Tris, tris(hydroxymethyl)aminoethane; MES, 2-(N-morpholino) ethanesulfonic acid; HEPES, 2-(4-(2-hydroxyethyl)piperazin-1-yl) ethanesulfonic acid; ATPγS, adenosine 5′-O-(thiotriphosphate); ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; EtBr, ethidium bromide; MANT, 2′-3′-O-(N-methylanthraniloyl); FRET, fluorescence resonance energy transfer.

☆ This research was supported by NSF RUI-0814716 and NSF-REU 1062629.

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http://dx.doi.org/10.1016/j.bpc.2013.08.001

1. Introduction

RecA is a naturally aggregating *Escherichia coli* protein integral to the maintenance of genetic integrity. RecA performs DNA repair, catalyzes the DNA strand-transfer reaction in homologous recombination, and initiates the bacterial SOS response [1]. RecA has a pI of 5.6, and is an allosterically regulated DNA and ATP binding protein that forms distinct oligomeric structures, and has also been extensively studied using a variety of techniques including in-depth analysis of multiple RecA mutants [2,3]. In solution, RecA adopts a mixture of higher-order structures including oligomers, rods, aggregated filaments and bundles, the distribution of which is dependent on protein concentration, cofactors, and solution conditions [4–6]. Previous work in our laboratory has investigated the thermal stability, unfolding transitions and aggregation of RecA in the presence of different ions found in the Hofmeister series. These experiments were performed in high concentrations of salts ranging from 250 mM to 2 M. The previous experiments showed that the unfolding transitions, thermal stability and aggregation states were ion and concentration dependent. RecA showed a reverse-anionic Hofmeister thermal stabilization that may likely arise from specific ion binding, changes in interfacial tension, and altered protein–solvent interactions that can give rise to unique, thermally stable RecA aggregates [7].

The literature reveals some studies that indicate buffers can alter biochemical properties although it is usually assumed that buffering agents will not act as a substrate or denaturant or alter biochemical properties. Norman Good and colleagues suggested several considerations for choosing buffering agents, including pK_a, solubility, and enzymatic inertness [8,9]. Much of Good's work concerned buffering agents' temperature sensitivity and interference with biochemical assays. More recently, Ugwu and Apte reviewed buffer effects on protein conformational stability and a variety of other effects on biochemical systems [10]. A review of the literature cited therein suggests more investigation into the interaction between buffer and protein may be necessary for optimization of biochemical systems in solution. Another example in the literature used dynamic light scattering (DLS) studies on IFN-τ to show that buffers differentially altered the thermally-induced protein aggregation and found larger aggregates formed in decreasing order of phosphate, Tris, and histidine [11]. Studies on the aggregation of humanized IgG found that phosphate, citrate, MES, MOPS and imidazole buffers formed decreasing percentages of aggregates following thermal denaturation that the authors suggest may be a result of decreasing solution ionic strength [12]. A study on the restriction enzyme *Eco*RV found decreasing reaction rates from HEPES to TES to Tris buffers associated with the availability of the amino group to interfere with protein cation–DNA phosphate interaction [13]. A study of *E. coli* inorganic pyrophosphatase found that buffers may alter Mg^{2+} affinity, substrate binding and quaternary structure [14]. These studies and others show that buffers can influence important biochemical properties of proteins. Interestingly, preliminary data from past work in our laboratory suggested that significant buffer dependent differences in RecA thermal stability occur even at low buffer concentrations of 20 mM.

In this work we have investigated the influence of four common biological buffers on RecA's thermal stability, activity, and substrate binding. RecA is interesting as it is a naturally aggregating protein whose function is dictated by the structure of the nucleoprotein filament and the bound cofactors. More recently RecA has been used in some interesting nanoscience applications such as using porous vesicles to modulate interactions between RecA and DNA [15] and using RecA filaments to synthesize conductive metal nanowires [16]. Previous studies in our lab have identified high salt conditions (250 mM–2 M) that stabilize RecA complexes and make them more resistant to thermal denaturation [7]. Here we show that simply altering the buffering agent or pH can also stabilize RecA and use of these conditions may be more applicable for additional nanoscience applications.

We used circular dichroism to monitor the structure and aggregation of RecA during thermal unfolding experiments in varying pH and buffer conditions. Circular dichroism and infrared spectra show only subtle differences and suggest very minor, if any, changes in the overall protein structure are associated with altering the buffer composition. Fluorescence experiments show distinct rates of dsDNA nucleation of RecA. Interestingly, as compared to the work exploring salt effects on the thermal stability and aggregation of RecA, this work was performed on RecA in 20 mM concentrations of buffers, significantly lower than 250 mM–2 M salt concentrations used previously. The present work provides sufficient evidence that buffers, independent of pH, have minimal effects on nucleotide binding but have significant effects on the thermal stability, unfolding transitions and dsDNA nucleation. However, this work also shows that increasing pH can also increase RecA's thermal stability and alter the unfolding transitions. Thus, altering the buffer or pH are yet other solution conditions to consider for researchers involved in studying the complex interactions between protein stabilization, misfolding and aggregation.

2. Methods

2.1. Preparation of RecA

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 Amicon Ultra 0.5 mL (10 kD) centrifugal concentrator. Protein was stored at −20 °C until use.

2.2. Circular dichroism studies of thermal unfolding

RecA was diluted into the previously described buffer, or into 20 mM HEPES, MES, or phosphate buffers all containing 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM $MgCl_2$. Thermal unfolding of RecA was performed in HEPES, Tris, or phosphate buffers at pH 6.5, 7.0, 7.5, 8.0 and 8.5 although experiments in MES were limited to pH 6.5 and 7.0 due to its low pK_a. Most CD experiments were performed with a final RecA concentration of 5 μM. For experiments with cofactors 12.5 μM ATP and/or 12.5 μM ATP ssDNA were added and the RecA concentration was 2.5 μM. Most CD experiments were performed using a 1 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. Fluorescence experiments and CD experiments with ATPγS and/or ssDNA used a 3 mm path length cell from Starna Cells. Nitrogen flow rate was purged at 100 mL/min for all CD and fluorescence experiments to minimize noise from HEPES and MES absorbance in the far-UV region. 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 for 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. For all experiments, 222 nm voltages were within accepted limits (<700 V at 216 nm and significantly lower for 222 nm), which allowed for monitoring of thermally-induced loss of secondary structure by plotting ellipticity at 222 nm versus temperature. These plots were used to monitor transitions and obtain melting temperatures under all conditions. Turbidity or the HT voltage at 285 nm was monitored on identical samples to obtain information on protein aggregation [17].

2.3. ATR-IR studies of RecA

Infrared spectra of RecA were obtained in pH 7.0 buffers using a Bruker Vertex 70 ATR-IR with LN-MCT detector by co-adding 500

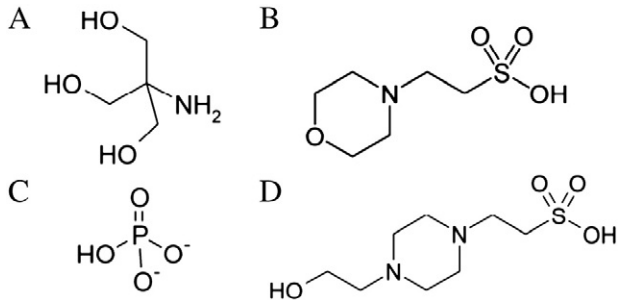

Fig. 1. Structures of (A) Tris, (B) MES, (C) phosphate and (D) HEPES buffers used to study RecA thermal stability and nucleotide binding.

scans (velocity, 20 kHz; apodization, Happ-Genzel; resolution, 4 cm^{-1}; phase resolution, 32; phase correction, Mertz). All infrared samples contained a final RecA concentration of 50 μM in the aforementioned buffers. Spectra were obtained at 25 °C, following a 60 minute period allowing protein to settle on the crystal. The buffer absorbance spectra were subtracted from protein spectra by attempting to eliminate the 2125 cm^{-1} water peak as Rahmelow et al. suggest [18] while working to have a flat baseline in the 1750–2000 cm^{-1} region as suggested by Dong and Lam [19].

2.4. 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 (Invitrogen Corp.). Fluorescence was monitored in step scan mode at an emission wavelength range (λ_{em}) of 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 excitation wavelength (λ_{ex}) of 270 nm while direct excitation of the MANT nucleotide was achieved at λ_{ex} of 356 nm. Data were collected at 25 °C following a 2 minute equilibration. Unfolding data were obtained in 5 °C intervals between 35 °C and 55 °C by allowing samples to equilibrate for 2 min at each temperature, lowering the temperature to 25 °C for 2 min and collecting data.

2.5. Ethidium bromide DNA nucleation assays

Nucleation of RecA on double-stranded DNA (dsDNA) was monitored in each of the different buffers at pH 7.0 using ethidium bromide fluorescence as described in [20]. Fluorescence was monitored using a Jasco FP-8500 Spectrofluorometer in single emission mode at λ_{em} of 595 nm with an λ_{ex} of 510 nm over 1 h (bandwidth, 5 nm; response time, 1 s; data interval, 1 s; sensitivity, low). Background fluorescence of buffer with ethidium bromide in the absence of DNA was subtracted from the fluorescence intensities. All solutions contained 4 μM RecA, 22 μM dsDNA (base pairs) (Sigma) and 2 μM ethidium bromide; dsDNA nucleation was initialized with the addition of ATPγS to a final concentration of 200 μM.

2.6. ATP hydrolysis assays

ATP hydrolysis was monitored using an enzyme-coupled spectrophotometric assay similar to that described by [21] with minor modifications. All assays were monitored using a Perkin Elmer Lambda 35 UV/Vis spectrophotometer with an 8 cell changer apparatus at 37 °C. Assays were performed in all buffer/pH combinations with 2.0 mM phospho(enol) pyruvate, 1.0 mM $MgCl_2$, 25 units/mL pyruvate kinase and L-lactate dehydrogenase, 0.15 mM NADH, 1.0 μM RecA and 10 μM poly(dT) (Sigma). Rates of ATP hydrolysis were calculated using ΔA_{340} nm per second with $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$ for NADH [21].

3. Results

3.1. Circular dichroism studies of RecA unfolding in different solution conditions

Fig. 1 shows the structures of the different buffers used for all of the studies. Fig. 2A shows the CD spectra of 5 μM RecA in the four buffers (20 mM) examined at pH 7.0 at 25 °C. The overall shapes of the CD spectra are quite similar in all of the buffers with small differences in intensity at 222 nm at pH 7.0. The turbidity measurements of the voltage (HT) signal at 285 nm are nearly identical (Fig. 5B and Supplemental data) suggesting similar oligomerization states for the different buffers [17,7]. The infrared spectra (Fig. 2B) do not show any substantial shape changes that are dependent upon the buffer or any changes that would suggest altered oligomerization states of the RecA protein. The Tris buffer does show a minimal increase in the relative intensity of the1635 cm^{-1} vibration, yet this is a very small change. Thus the CD and infrared data suggest that the overall secondary structure of RecA is not significantly altered by changes in buffer composition at concentrations of 20 mM buffer. Fig. 3 shows an overlay of the CD spectra of RecA obtained in Tris buffer at different pH conditions. This data also shows that there are not major pH dependent secondary structural changes in the protein. The turbidity measurements of the voltage

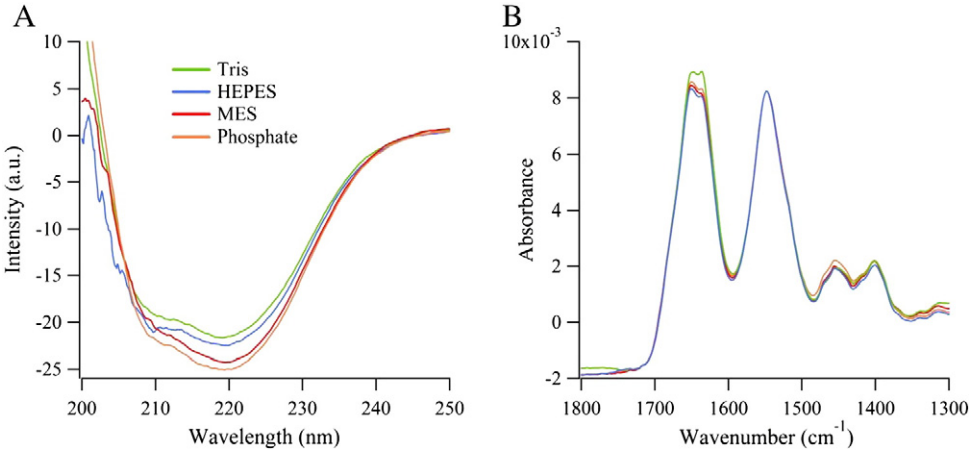

Fig. 2. (A) CD spectra of 5 μM RecA in 1 mm cell and (B) ATR-FTIR spectra of 50 μM RecA in either 20 mM Tris, HEPES, MES or phosphate (pH 7.0) each containing 1 mM DTT, 1 mM $MgCl_2$ and 0.1 mM EDTA at 25 °C.

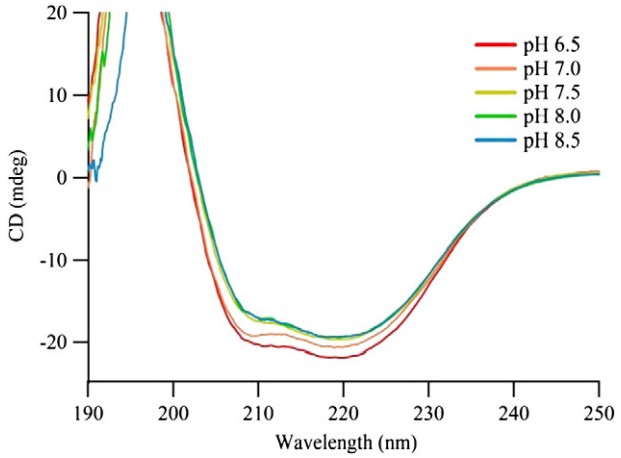

Fig. 3. CD spectra of 5 μM RecA in 1 mm cell at 25 °C in 20 mM Tris buffer containing 1 mM DTT, 1 mM $MgCl_2$ and 0.1 mM EDTA at pH 6.5, 7.0, 7.5, 8.0 and 8.5.

(HT) signal at 285 nm are within 4 voltage units for pH 6.5–8.5 in Tris buffer (data not shown). The Supplemental data also shows that increasing pH does not significantly influence the overall secondary structure of the protein in any of the buffers used for this study. CD intensities at 222 nm were plotted as a function of temperature and used to determine the melting temperatures present in Table 1.

Although the CD and infrared data do not show significant changes, Fig. 4 clearly shows that the buffers dramatically influence the melting profiles of RecA at pH 7.0. Inspection of the unfolding profiles shown in Fig. 4 also revealed buffer differences in the RecA unfolding transitions. All unfolding profiles were performed on at least two separate RecA samples and usually showed melting temperatures within about 2 °C with a maximum of about 5 °C difference between profiles. Although phosphate (Fig. 4B), HEPES (Fig. 4C) and MES (Fig. 4D) all show initial loss of signal at 222 nm and more signal retained at 208 nm, the HEPES and MES buffers show more overall signal loss at the lower temperatures while RecA in phosphate shows only minimal loss of signal around 225 nm. MES unfolding transitions more closely resemble the phosphate transitions while HEPES profiles are intermediary between Tris and MES transitions (Supplemental data). Fig. 5A shows plots of ellipticity at 222 nm as a function of temperature for each of the buffers at pH 7.0. Similar plots for other pH values are found in the Supplemental data. These plots were used to estimate the melting temperatures for each of the conditions in Table 1. These data show that RecA is stabilized in increasing order by Tris < HEPES < MES < phosphate. Fig. 5B shows plots of turbidity or HT voltage at 285 nm as a function of temperature and reflects changes in aggregation of the protein [17]. The initial turbidity of all the samples is very similar and suggests minimal differences in oligomerization but also shows that after protein unfolding there is nonspecific aggregation that is reflected in increased turbidity (Fig. 5B). The decreased turbidity at higher temperatures, after unfolding transitions, may result from the precipitation of some of the nonspecifically aggregated protein. The unfolding data is summarized in Table 1 that shows that the phosphate buffer stabilizes RecA at every pH studied and does not completely unfold even at temperatures as high as 105 °C. HEPES and Tris buffers show 40–50 °C increases in melting temperature from pH 6.5 to 8.5.

Table 1
The melting temperatures of 5 μM RecA in 20 mM buffers containing 1 mM DTT, 1 mM $MgCl_2$ and 0.1 mM EDTA. Values were determined by plotting 222 nm ellipticity versus temperature.

pH	Melting temperature (°C)			
	Tris	MES	HEPES	Phosphate
6.5	55	65	65	>105
7.0	65	88	78	>105
7.5	73	ND	100	>105
8.0	87	ND	>105	>105
8.5	>105	ND	>105	>105

3.2. Fluorescence studies of nucleotide binding and DNA nucleation

In order to determine if the buffers were influencing nucleotide binding we performed a variety of studies using fluorescently-labeled ADP. These studies allowed us to determine how the different buffers alter MANT-ADP binding. In addition, we were able to determine if nucleotides were able to bind to partially unfolded RecA states. Fig. 6 shows the results of the fluorescence studies on RecA and MANT-ADP. In these studies the RecA was excited at 270 nm and emission of MANT-ADP at 440 nm was monitored. Transfer of energy from tyrosine residues in the ADP binding site of RecA results in a decreased tyrosine emission at ~340 nm and transfer of energy to the MANT-ADP residue, with emission at ~440 nm. Therefore, only MANT-ADP that is bound to RecA should result in fluorescence at 440 nm. These spectra were obtained after incubation at various temperatures from 25 to 55 °C and then samples were returned to 25 °C for a 2 minute equilibration period before monitoring the fluorescence spectra. The samples were returned to 25 °C in order to ensure that any changes observed were due to changes in nucleotide binding to partially unfolded states and not due to temperature dependent changes in protein or MANT fluorescence. CD spectra were obtained at 25 °C after each increase in temperature to ensure that the RecA did not refold. At 25 °C Tris, HEPES and MES all show similar amounts of fluorescence while the phosphate buffer results in 0.02 units less. Binding of the MANT-ADP is similar from about 25–40 °C with significant reduction in binding occurring at 45 °C, in HEPES, MES and phosphate buffers. The RecA in Tris buffer shows that more substantial binding is retained at 45 °C as compared to the other buffers. Clearly the buffers have minimal effects on RecA's ability to bind MANT-ADP and the results at different temperatures may suggest that the buffers induce small changes in unfolding transitions.

Ethidium bromide (EtBr) displacement experiments were performed in order to study how buffers influence RecA's ability to nucleate and bind dsDNA. Formation of an active RecA–ATPγS–DNA complex results in EtBr displacement from dsDNA and decreased fluorescence. We monitored the change in fluorescence over time, to investigate RecA's ability to nucleate and bind dsDNA in the different buffer conditions. Under all buffer conditions we observe a decrease in fluorescence over time that indicates that RecA is able to bind dsDNA and displace the EtBr. Fig. 7A shows results for experiments performed in 20 mM buffers and shows that in the presence of Tris buffer RecA is able to immediately nucleate on DNA and rapidly displace the EtBr. However, RecA in either 20 mM HEPES or MES buffer shows an intermediate nucleation rate and some remaining fluorescence after 1 h. Phosphate buffer significantly slows the EtBr displacement but results in nearly complete displacement of EtBr after 1 h. Fig. 7B shows that increasing buffer concentrations to 100 mM slows nucleation and decreases the overall EtBr displacement for all buffers relative to that observed at 20 mM (Fig. 7A).

3.3. Circular dichroism studies of unfolding with RecA cofactors

Fig. 8 summarizes the results obtained on the unfolding of RecA in the presence of ATPγS and/or ssDNA. Fig. 8A shows the CD spectra of RecA unfolding in the presence of ssDNA and ATPγS. Interestingly the presence of the cofactors results in unfolding transitions with significant signal retention at 208 nm and initial loss of signal around 225 nm that is similar to that observed in Fig. 4. Fig. 8B shows that the melting profile in the presence of ATPγS is nearly identical to the control and shows a large transition around 75 °C. Preliminary data obtained in the presence of ATP or ADP show results similar to that obtained with ATPγS. The RecA–ssDNA and RecA–ssDNA–ATPγS complexes show similar melting

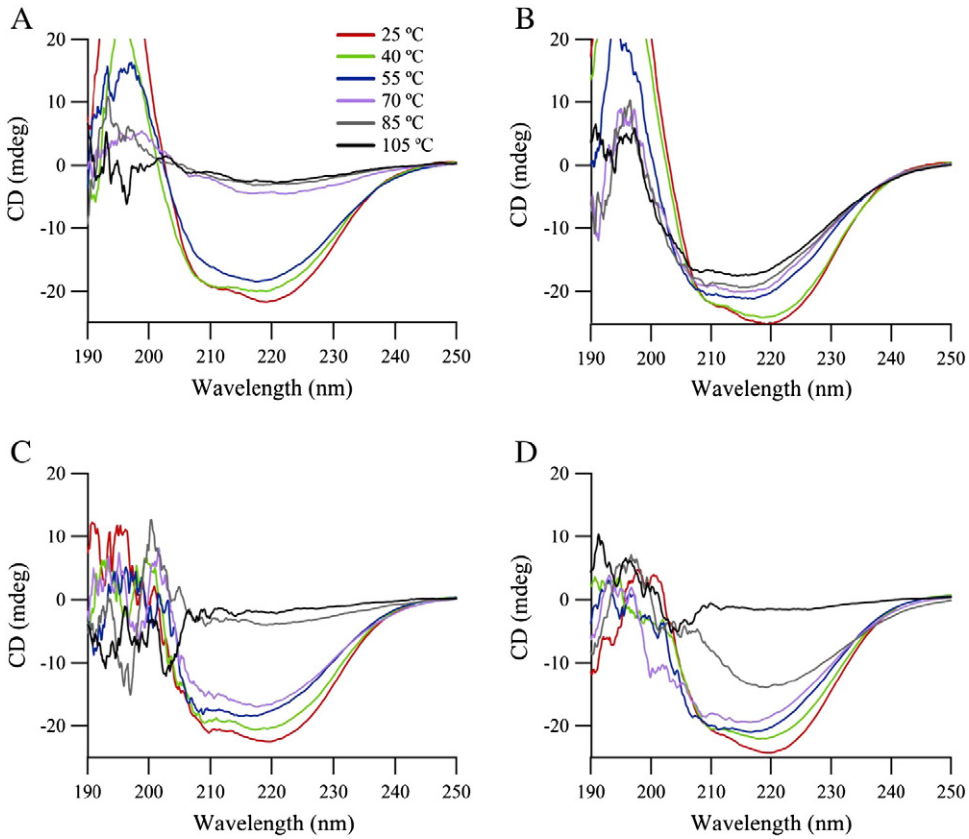

Fig. 4. Circular dichroism spectra of 5 μM RecA unfolding (25–105 °C) in 1 mm cell at pH 7.0 (A) Tris, (B) phosphate, (C) HEPES and (D) MES buffers each containing 1 mM DTT, 1 mM $MgCl_2$ and 0.1 mM EDTA. Each panel shows spectra obtained at 25, 40, 55, 70, 85 and 105 °C.

profiles and reveal large increases in the melting temperature similar to some of the other buffers.

4. Discussion

The studies presented here reveal that RecA's stability and unfolding transitions can be dramatically influenced by both changes in pH and buffer reagents. Interestingly, both the CD and infrared spectra obtained with RecA in the different buffers do not show significant changes in the overall secondary structure of the protein that could explain the significant increases in thermal stability. Furthermore, the turbidity and infrared data did not show any differences that would suggest significant changes in oligomerization states in the different buffers. This is evident as previous work showed that although NaCl, $MgSO_4$ and control RecA samples at 25 °C had turbidity differences around 10 units, dynamic light scattering showed no changes in the hydrodynamic diameter [7]. However the same studies showed large changes in turbidity and hydrodynamic diameter as unfolding and nonspecific aggregation occurred [7]. The turbidity differences observed in the present study are usually less than 5 units. In fact, neither changes in pH or buffer result in large changes in the overall CD spectra or turbidity. It is possible that small changes in RecA oligomers are not observable in DLS or turbidity measurements. The small differences in CD intensity may be due to slight differences in concentrations of the samples. However,

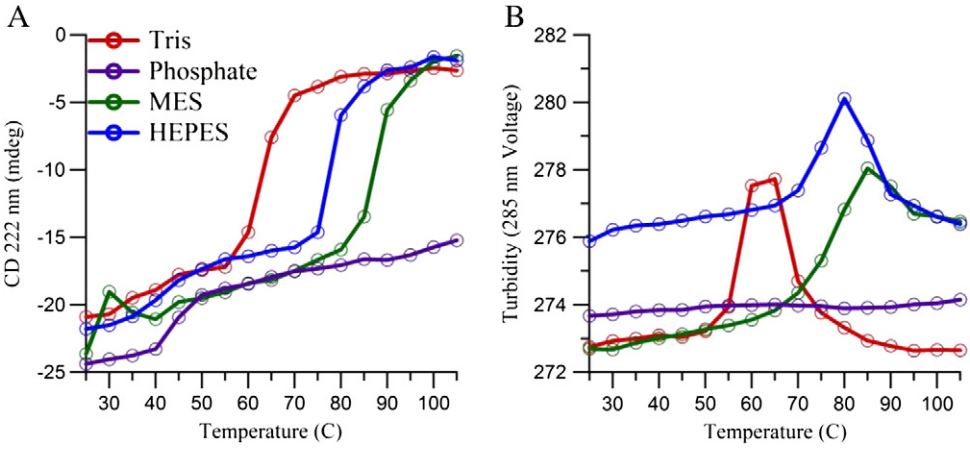

Fig. 5. Plots of signal intensities as a function of temperature of 5 μM RecA in pH 7.0 buffers. (A) 222 nm CD intensities and (B) HT voltages at 285 nm.

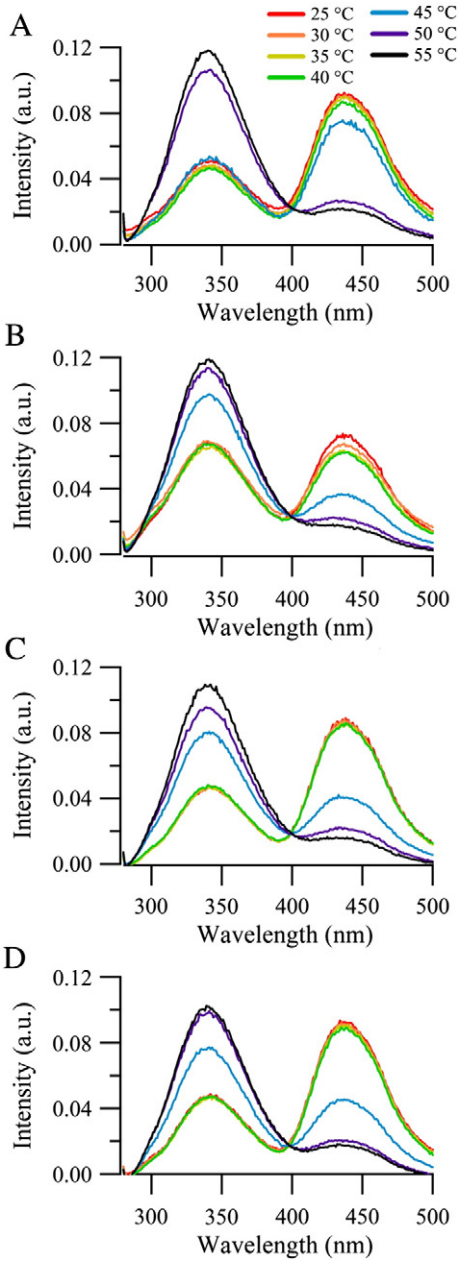

Fig. 6. Fluorescence studies of MANT-ADP binding to RecA in pH 7.0 (A) Tris, (B) phosphate, (C) HEPES and (D) MES buffers. Emission at 440 nm results from transfer of energy from excited tyrosine residues ($\lambda_{ex} = 270$ nm, $\lambda_{em} = 340$ nm) to RecA-bound MANT-ADP.

the unfolding studies show that increasing pH levels, in all of the buffers studied, resulted in large changes in RecA stability. Some of the buffer and pH combinations result in conditions such that RecA is resistant to complete thermal denaturation. Previous studies have shown that RecA's disassociation from ssDNA increases with increasing pH and is maximal at pH > 7.5 [22]. The amino terminal of RecA is more positively charged and interacts with the more negatively charged C-terminal of the neighboring monomer in assembled RecA nucleoprotein filaments [23]. Studies have shown the N-terminal domain is important for oligomer formation, facilitating RecA–ssDNA complexes and capturing the donor dsDNA and that N-terminal Lys and Arg residues are crucial for these functions [24–27]. Interestingly, decreasing positive charge on RecA seems to alter oligomer association and function yet we show here that increasing pH results in dramatic increases in RecA stability.

In addition to the pH studies, a complete set of experiments was performed to monitor RecA unfolding transitions and stability in the different buffers: Tris, phosphate, HEPES and MES. Tris is the most common buffer used in biochemical studies of RecA and was previously used to study how salts influence RecA stability, aggregation and unfolding [7]. The RecA unfolding transitions observed in previous experiments where chloride stabilized RecA complexes show a significant amount of signal retention at 216 nm, suggesting a stabilized β-structure [7]. However, as a general summary of the pH and buffers studied in this work we observe more stabilized alpha-helical structures at higher temperatures (Fig. 4 and Supplemental data). Previous CD studies on urea-induced unfolding of RecA ascribe some loss of signal around 222 nm to the unfolding of the N-terminal domain and they also suggest that a decrease in ellipticity around 222 nm was associated with disassociation of the oligomer [25]. Other studies suggest that the thermal stability of RecA and homologs arises primarily due to stabilization of the alpha-helical structures [28]. Helical content being associated with stability is consistent with the fact that in our data both phosphate (Fig. 4B) and MES (Fig. 4D) unfolding profiles show minimal loss around 208 nm as compared to Tris (Fig. 4A) and HEPES (Fig. 4C). These results suggest that 20 mM concentrations of buffers result in the retention of alpha-helical structures associated with increased thermal stability (Table 1). Previous studies showed RecA is stabilized in high concentrations of chloride salts and led us to investigate if increasing pH would stabilize the protein. In order to further investigate the mechanism of protein stabilization in these buffer conditions, additional experiments were performed to monitor how solution conditions influence ATP and DNA binding.

Interestingly, thermal stabilization of RecA in the different buffers is not associated with any significant differences in the ATP hydrolysis activity at pH 7.0 or 7.5 (data not shown). Nucleotide binding to RecA is only slightly influenced by the presence of phosphate buffer with nearly no effect from HEPES or MES on MANT binding to RecA at pH 7.0. However, as temperature is increased nucleotide binding to RecA in Tris is increased as compared to binding in HEPES, MES and phosphate buffers. This suggests that at 45 °C the nucleotide binding site is more intact or more accessible in the Tris buffer as compared to the other buffers. Although the buffers do not significantly impact nucleotide binding their presence most definitely influences RecA's ability to bind dsDNA. Fig. 7A shows the decrease in EtBr fluorescence over time as RecA–ATPγS complexes nucleate on dsDNA–EtBr complexes and displace the EtBr from the DNA. Increased RecA stability is inversely correlated with DNA binding. HEPES and MES decrease the nucleation rate and do not allow RecA to completely displace the EtBr after 1 h. However, the presence of the phosphate buffer further slows nucleation but results in more complete displacement after 1 h (Fig. 7A). Fig. 7B shows that increasing the buffer concentrations decreases displacements and suggests that the buffers may compete with initial DNA binding with phosphate being the most effective. Pugh and Cox suggested that high salt, DNA-independent ATPase activity may be due to the anions in solution mimicking the negative charges of the DNA backbone [29]. The phosphate buffer most certainly could interact with RecA where it binds DNA and increase stability while competing with dsDNA binding. HEPES and MES may interact with RecA in a slightly different fashion that alters RecA complex formation or alters the equilibrium between monomers and oligomers resulting in slower nucleation.

The possibility exists that the buffers interact with RecA at multiple sites and influence protein stability and DNA binding but do not significantly alter ATP binding as evidenced by the ATP hydrolysis and fluorescence studies. Studies on *Eco*RV found that buffers had large effects on the reaction velocity by altering protein–DNA binding [13]. The authors found that Tris buffer screened DNA phosphates and prevented binding to *Eco*RV while the presence of HEPES did not screen the DNA and resulted in increased activity [13]. Our results are the opposite of those obtained on *Eco*RV and suggest that the anionic buffers,

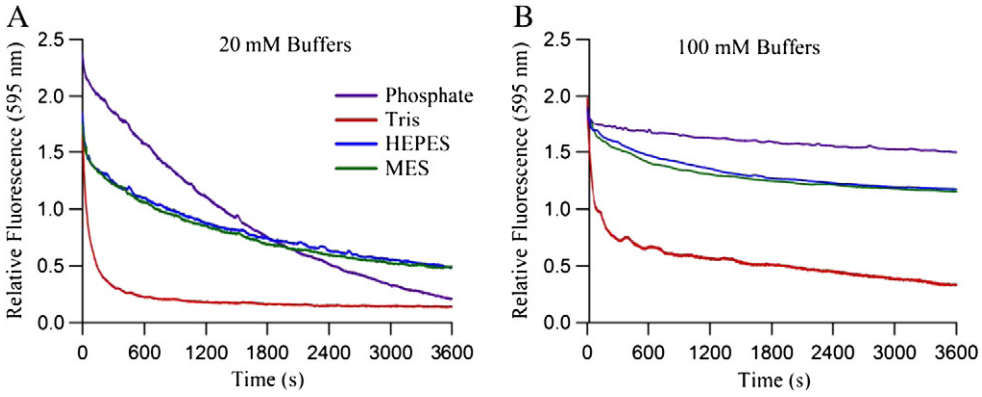

Fig. 7. Fluorescence assays of EtBr displacement by RecA in pH 7.0 buffers. All reactions contained 4 μM RecA in either 20 mM Tris, HEPES, MES or phosphate each containing 1 mM DTT, 1 mM $MgCl_2$, 0.1 mM EDTA and 22 μM dsDNA (base pairs), and were initiated with 200 μM ATPγS at 37 °C.

HEPES, MES and phosphate do slow RecA's nucleation on dsDNA and may be interacting with the protein or altering small changes in the monomer–oligomer distribution. Previous experiments in our lab aimed at investigating conditions used for difference infrared studies have shown that activity assays performed in 100× HEPES buffer allowed RecA to hydrolyze ATP in the absence of DNA and suggested that high concentrations of HEPES may mimic DNA binding [30]. NMR studies also provide additional evidence to suggest protein–buffer interactions may be responsible for the increases in RecA stability at low buffer concentrations. NMR studies performed on human liver fatty acid binding protein (hLFABP) show that as MES concentration is increased from 0 to 50 mM some amino acid residues had changes in chemical shifts [31]. These studies revealed that MES has weak interactions with polar and nonpolar amino acids that did not alter the overall tertiary structure [31]. However, MES and Bis-Tris buffers did result in significant changes in hLFABP's dynamics [31]. NMR studies performed on the C-terminal Src homology 2 (SH2) domain of phospholipase C-γ1 (PLCC SH2) revealed specific interactions between buffer phosphate and Arg side chains [32]. Comparison of NMR data obtained in phosphate and histidine buffers showed that the buffer phosphate interacts with arginines in the phosphopeptide binding pocket [32]. The combination of these studies provides further evidence that buffer–protein interactions could be responsible for the differences in RecA stability in the different buffers at low concentrations.

Overall for all of our experiments we observe that the decrease in positive charge on the protein is associated with increased RecA stability. In addition we see more dramatic increases in stabilization of RecA when the pH is increased to a pH above the pK_a of the respective buffer. Ugwu and Apte review buffer effects on stability and suggest increased stabilization if anionic buffers are used above the pI [10]. MES, HEPES and phosphate all have negative charges above their pK_a with the most stabilizing buffer, phosphate, having the largest amount of negative charge (Fig. 1). Interaction of negatively charged buffers with RecA would result in increasing the overall net negative charge on RecA and thus would correlate with increasing pH enhancing RecA stability. At low pH where the protein has the most positive charge we observe the largest dependence on buffer identity yet as we increase pH there is an overall stabilization that is less dependent on the buffer composition. Additional evidence to support buffer–RecA interactions arises from the direct correlation of the increased stability and decreased ability to nucleate on dsDNA. Furthermore, the thermal unfolding of RecA in the presence of DNA results in increased stabilization and intermediate unfolding states that resemble those obtained in the most stabilizing buffers (compare Fig. 7A with Fig. 4B & D) and are very different from those obtained in the presence of stabilizing salts [7].

The results of this paper show that the choice of buffers may be more important than many biochemists might infer. Furthermore, major differences in stability of the protein may not be reflected in typical activity assays or methods used to study protein secondary structure or nucleotide binding. These studies may be of general interest to those working with DNA binding proteins as it is clear that small alterations in solution conditions can dramatically influence stability. The conditions presented in this paper may be of use for those using RecA for nanoscience applications. Importantly, altering the pH or buffering reagent can dramatically increase RecA stability and make it even more amenable for additional applications. These studies show that the choice of buffer may also be very important in the study of protein misfolding diseases as the buffer conditions may alter stability and aggregation without changing the overall structure.

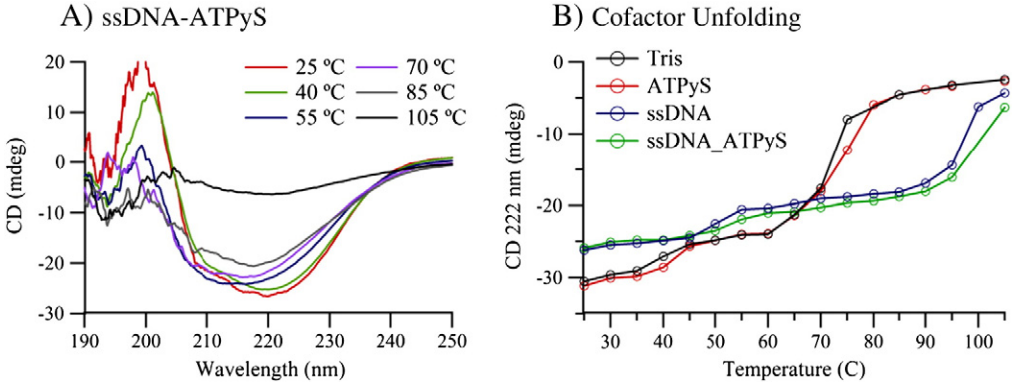

Fig. 8. (A) Circular dichroism spectra of 2.5 μM RecA unfolding in 3 mm cell in the presence of 12.5 μM ssDNA (poly(dT)) and 12.5 μM ATPγS. (B) Plots of 222 nm CD intensities as a function of temperature of 2.5 μM RecA in pH 7.0, 20 mM Tris buffer with 12.5 μM ssDNA (poly(dT)) and/or 12.5 μM ATPγS.

Acknowledgments

We would like to acknowledge Dr. Yanjie Zhang and Dr. Chris Berndsen for their helpful discussions about the work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bpc.2013.08.001.

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