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Probing Buffer-specific Effects on Nucleotide Binding to RecA using Difference Fourier Transform Infrared Spectroscopy

Joshua E. Temple
James Madison University

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Probing Buffer-specific Effects on Nucleotide Binding to RecA using Difference Fourier Transform Infrared Spectroscopy

An Honors Program Project Presented to
the Faculty of the Undergraduate
College of Science and Mathematics
James Madison University

by Joshua Edwin Temple
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FACULTY COMMITTEE:  HONORS PROGRAM APPROVAL:

Project Advisor:  Gina MacDonald, Ph.D.,
Professor, Biochemistry and Biophysics

Reader:  Yanjie Zhang, Ph.D.
Assistant Professor, Physical Chemistry

Reader:  Christopher Berndsen, Ph.D.
Assistant Professor, Biochemistry

PUBLIC PRESENTATION

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Abstract

The *Escherichia coli* protein RecA catalyzes the strand exchange reaction used in DNA repair and genetic recombination. RecA is also a target for inhibiting microbial antibiotic resistance, understanding cancer propagation, and characterizing neurodegenerative disorders. Therefore, understanding factors that affect RecA structure and stability is broadly applicable to many fields. Previous studies in our lab have shown buffer-specific changes in RecA stability and unfolding transitions. These studies suggest only minimal buffer-dependent changes in nucleotide binding and secondary structure but do not explain the significant differences in RecA stability and unfolding profiles. Here we have employed various biochemical and spectroscopic techniques to further characterize RecA both structurally and functionally in four common buffers: Tris, MES, HEPES, and Phosphate. Activity assays reveal RecA activity may be slightly decreased in HEPES and Phosphate buffers as compared to Tris and MES. Circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy show conservation of global RecA secondary structure in all buffers, yet unique aggregation states are observable in CD turbidity plots. Laser-induced photolysis of caged nucleotides was also used in conjunction with difference FTIR spectroscopy to generate RecA-ADP minus RecA difference infrared spectra in each of the four buffers. These studies detected unique buffer-specific changes in nucleotide binding to RecA in each buffer and may provide insight into mechanisms for buffer-specific stability profiles of RecA.
Introduction

The *Escherichia coli* protein RecA serves in a variety of cellular functions including facilitating DNA strand exchange during homologous recombination, mending stalled replication forks, and acting as a co-protease for the LexA repressor to initiate the bacterial SOS response.\(^1\) These processes in turn act to preserve genomic integrity of the organism and ensure survival.\(^2\) RecA is highly self-associative and therefore forms filamentous structures in order to carry out these functions. Furthermore, RecA has well-conserved homologues in almost every domain of life, thus establishing it as an ideal model protein to study in a variety of contexts.\(^1\)–\(^4\) For example, certain forms of cancer are associated with defects in DNA recombination.\(^5\) A more thorough characterization of protein aggregation will also aid in understanding various neurodegenerative disorders.\(^5\) Further research into elucidating how microorganisms propagate antibiotic resistance and inhibiting cellular machinery like RecA responsible for this process serves as a target for improving antibiotic efficacy.\(^6\) Therefore, these studies concerning RecA structure and stability are broadly applicable to many fields.

RecA is a 37.8 kDa protein comprised of 352 amino acids and has been crystallized in both monomeric and polymeric forms, including an active filament, a RecA-ADP complex, and a hexamer aggregate.\(^2,7\)–\(^11\) RecA binds single-stranded DNA (ssDNA) to form a nucleoprotein filament, facilitated by van der Waals and electrostatic contacts between the RecA L1/L2 loops and ssDNA (Figure 1, red).\(^2\) RecA polymerization onto ssDNA is highly cooperative and requires ATP binding to the Walker A and B motifs at monomeric interfaces.\(^12\)–\(^14\) Subsequent ATP hydrolysis and strand exchange reactions are allosterically coupled, ultimately resulting in filament disassociation.\(^2,12,15\)–\(^17\) The N-terminus of RecA is essential for this oligomerization at high RecA concentrations or with DNA present, causing RecA to take on an extended
conformation, whereas C-terminal interactions are vital for DNA binding and nucleoprotein filament formation (Figure 1B, C, D). High salt concentrations have also been shown to induce an extended RecA conformation similar to an active RecA-ssDNA nucleoprotein filament, stimulating ATPase activity.

![Image](71x291 to 539x621)

**Figure 1.** (A) Crystal structure of the RecA-ADP complex (PDB: 1REA). (B) Representative homotrimer of RecA bound to ssDNA (black) (PDB: 3CMU). (C) Surface representation of (B). (D) Back view of (C). For all structures: ADP, black sticks; L1/L2 loops, red; Lys72 and Thr73 of the Walker A motif, yellow; catalytic residues (Glu96, Lys248, Lys250), orange; oligomeric contacts (Glu38, Lys216, Phe217, Arg222, Ile298), magenta.

Previous studies also implicate various solution conditions such as ionic strength, pH, and buffer molecules as playing a significant role in altering RecA structure, ATPase activity, and stability. However, the causes are not immediately clear. Here we seek to understand the cause for vast thermal stability differences of RecA in four common buffers: Tris, MES, HEPES,
and Phosphate (Figure 2). Protein chemistry dictates careful choice of buffer when considering proper experimental design. Good et al. suggest multiple criteria for proper buffer selection, including optimal $pK_a$, structure, and lack of chemical activity. Improper buffer selection may have substantial effects on a system. Crystallography studies show buffering agents located at multiple sites in proteins, including the active site. Sulfates, phosphates, and other buffer molecules have been shown to potentiate or inhibit enzyme activity through direct interactions with the protein, altering substrate binding affinity and catalytic rate. Tris buffer along with other amine-containing buffers has also been postulated to interfere with biochemical studies in a variety of ways, including acting as an enzymatic substrate, competitive inhibitor, or activator as well as interacting with substrate directly. Buffer molecules are not thought to cause substantial global conformational alterations to protein structure, yet instead may induce more subtle structural effects. However, significant buffer-dependent changes in protein dynamics are evident that can affect activity and protein-protein interactions.

Figure 2. Molecular structures of Tris, MES, HEPES, and Phosphate buffers.

Therefore, the mechanisms by which buffers alter thermal stability could involve numerous factors. These factors could include differential buffer-protein stabilizing interactions, buffer-specific protein conformation and dynamics differences, and unique promotion of RecA to different oligomerization states. In order to investigate each buffer’s influence on RecA thermal stability, aggregation state, structure, and activity, we used various biochemical and
spectroscopic techniques. Previously it has been shown that buffer identity can affect RecA nucleation rates onto dsDNA.\textsuperscript{21} Förster resonance energy transfer (FRET) studies examining quenching of MANT-ADP energy by tyrosine residues in RecA’s binding site show similar quenching in each buffer, confirming a lack of globally significant differences in nucleotide binding.\textsuperscript{21} Here we further characterize RecA behavior in each buffer.

To gain biochemical insight into buffer influences on RecA function, a coupled pyruvate kinase-lactate dehydrogenase activity assay was used to probe the activity of RecA. A scheme of the assay is presented in Figure 3 in which 1 mol of ATP hydrolyzed by RecA corresponds to 1 mol of NADH oxidized in the sample. Thus, RecA ATPase activity may be monitored by following the loss of absorbance by NADH at 340 nm, providing insight into buffer-specific influences on RecA ATP hydrolysis. Activity changes in each buffer system can indicate differences in RecA oligomerization or nucleotide cofactor binding, suggesting an altered structure.

**Figure 3.** Schematic of the coupled enzyme RecA activity assay. RecA is maintained as the limiting reagent. Oxidation of NADH to NAD\textsuperscript{+} as monitored by loss of absorbance at 340 nm is related in a 1:1 ratio to ATP hydrolysis by the RecA-ssDNA nucleoprotein filament. Figure adapted from Metrick.\textsuperscript{47} (PDB: 3CMT).
Further spectroscopic techniques were employed to structurally characterize RecA in each buffer. CD and attenuated total reflectance (ATR) FTIR spectroscopy were used to evaluate any RecA secondary structural differences in each buffer. CD is a form of spectroscopy used to characterize protein secondary structure. A CD spectrum is the result of a sample’s differential absorption of left- and right-circularly polarized light, and the shape of each spectrum is dependent on protein secondary structure (α-helices or β-sheets). Primarily α-helical content is shown by characteristic double-minima at 208 and 222 nm, whereas predominantly β-sheet folds are shown by a single minimum between 210 and 220 nm (Figure 4). Upon protein denaturation, loss of secondary structure corresponds to a decrease in CD signal. To supplement CD, ATR FTIR is also a useful tool for observing protein structure, including secondary structure, quaternary structure, solvation, and protein backbone environment. In IR spectroscopy, spectra arise from chemical bond vibrations. The energy of the vibration is dependent upon the identity of the atoms contributing to the bond, which allows for the determination of specific vibrational features attributable to protein structure and environment.

Smaller scale structural differences in each buffer can be probed using difference FTIR spectroscopy in conjunction with laser-induced photolysis of caged nucleotides. Here, we have employed this method to monitor buffer-specific structural changes, as well as residues being perturbed, upon nucleotide binding to RecA. Past difference FTIR experiments have successfully
isolated individual amino acid changes related to electron transfer, proton pumping, and nucleotide binding to numerous proteins.\textsuperscript{31–36}

There are many characteristic regions of an FTIR spectrum that are widely used to evaluate these changes in protein structure, including the amide I, II, and III vibrations of chemical bonds. Jackson and Mantsch outline these vibrational modes and their variants.\textsuperscript{31} Approximately 80\% of the amide I vibration, located around 1620-1690 cm\textsuperscript{-1} depending on secondary structure, is due to C=O stretching with minor contributions from C-N stretching. Amide II, centered around 1550 cm\textsuperscript{-1}, arises from \textasciitilde60\% N-H bending and \textasciitilde40\% C-N stretching.\textsuperscript{31} Amide III vibrations are found from 1200-1350 cm\textsuperscript{-1} and arise from C-N and C-C stretching, N-H and C=O in-plane bending, and CH\textsubscript{2} wagging.\textsuperscript{31}

Furthermore, vibrations in the 1610-1628 cm\textsuperscript{-1} range have been linked to protein aggregation which is of special interest with RecA, a naturally aggregating polypeptide.\textsuperscript{31} There are also numerous vibrations that arise from amino acid side chains which will allow for the assignment of specific residues affected upon nucleotide binding to RecA.\textsuperscript{37} Certain vibrations are given in Table 1.

Past difference FTIR experiments using these methods have shown differential perturbations between ADP and ATP binding to RecA in Tris and HEPES buffers, but not MES.
or Phosphate.\textsuperscript{38,39} The method used to generate these difference FTIR spectra is shown in Figure 5, depicting the photolytic release of cage from caged-ADP. As a caged species, the nucleotide cannot bind to RecA. Binding is only permitted upon cleavage of the cage. This technique is invaluable in generating double-difference spectra with reproducibility in order to observe buffer-specific differences in RecA behavior.

\textbf{Figure 5.} Photolytic release of a 1-(2-nitrophenyl)ethyl group, or cage, from caged-ADP.

Previous studies have also shown numerous amino acids involved in nucleotide binding, including Asp100, Asp144, Ser145, and residues comprising the MAW motif including Thr42, Asp48, Ile61, Val62, Ile64, and Tyr65.\textsuperscript{1} Amino acids Glu96, Lys248, and Lys250 render RecA catalytically active.\textsuperscript{2,10} Asp100 has also been shown to govern nucleotide specificity.\textsuperscript{40} Mutations at Lys72 alter the protein so it can bind but not hydrolyze ATP, suggesting its role in ATP hydrolysis (Figure 1).\textsuperscript{41} By examining vibrational changes of various side chains upon nucleotide binding in each buffer, we will be able to determine the extent to which each buffer may influence RecA conformation and binding ability. These experiments can also serve as an indirect probe of aggregation. Aggregate formation causes slight changes in the environment of residues in each monomer, causing minor shifts in the IR spectrum. Thus, the conformational inhomogeneity contributes to poorer signal definition in difference spectra and results in broadened spectral features. Difference IR spectra generated in each buffer will allow us to investigate differences in nucleotide binding to better understand buffer-specific thermal stabilities from a structural perspective.
Experimental

Preparation of RecA

RecA was purchased from New England Biolabs (Ipswich, MA) and exchanged into pH 7.0 Tris (tris(hydroxymethyl)aminoethane), MES (2-(N-morpholino) ethanesulfonic acid), HEPES (2-(4-(2-hydroxyethyl)piperazin-1-yl) ethanesulfonic acid), or Phosphate buffer using Amicon Ultra 0.5 mL 10 kDa concentrators. All buffers contained 20 mM buffer, 1 mM dithiothreitol (DTT), 1 mM MgCl₂, and 0.1 mM EDTA. RecA aliquots were centrifuged at 12,000g and 4 °C for 1 hr periods. The resulting RecA concentrate was washed four times each with pH 7.0 respective buffer at 12,000g and 6 °C for 30 min. Protein concentration was determined using a Perkin Elmer Lamda Bio+ UV-Vis spectrophotometer using Beer’s Law (A₂₈₀ = 0.59). Aliquots of RecA were prepared for a final concentration of RecA appropriate for each experiment and frozen only once at -20 °C before use.

Activity Assays

RecA ATPase activity in the presence of ssDNA was monitored spectrophotometrically using a coupled pyruvate kinase-lactate dehydrogenase assay in which there exists a 1:1 relationship between ATP hydrolyzed and NADH oxidized (Figure 4). Loss of absorbance at 340 nm due to NADH oxidation was used to determine the rate of ATP hydrolysis. All trials contained 20 mM buffer at pH 7.0, 3.0 mM phospho(enol) pyruvate, 25 units/mL each of pyruvate kinase and lactate dehydrogenase, and 10 μM poly(dT), to which 0.32 mM NADH and 0.5 mM ATP was added to start the reaction. All reagents were kept in excess of RecA (1 μM). Both ATP and poly(dT) were purchased from Sigma whereas NADH, pyruvate kinase, and lactate dehydrogenase were obtained from Boehringer Manheim.
Assays were performed in a 1 mL, 1 cm Plastibrand cuvette (Sigma Aldrich) using a Perkin Elmer Lamda 35 UV/Vis spectrophotometer at room temperature. Absorbance changes were related to NADH concentration using $\varepsilon = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$. All trials were repeated in triplicate.

**ATR FTIR**

ATR 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 scans (velocity, 20 kHz; apodization, Happ-Genzel; resolution, 4 cm$^{-1}$; phase resolution, 32; phase correction, Mertz). All samples contained a final RecA concentration of 50 μM in each buffer. Spectra were obtained at 25 °C, following a 60 min period allowing protein to settle on the crystal. The buffer absorbance spectra were subtracted from protein spectra by flattening the 2125 cm$^{-1}$ water peak as Rahmelow et al. suggest$^{42}$ and attempting to eliminate vibrations around 1750–2000 cm$^{-1}$ as suggested by Dong and Lam.$^{43}$

**Circular Dichroism**

RecA in Tris buffer was thawed and added to Tris, HEPES, MES, or Phosphate buffer until a final RecA concentration of 5 μM in 150 μL was reached. All thermal stability CD analysis was conducted in a 1 mm path length quartz cell from Starna Cells (Philadelphia, PA). RecA unfolding in each buffer at pH 7.0 was conducted in 20 mM buffer, 1 mM DTT, 1 mM MgCl$_2$, and 0.1 mM EDTA.

A Jasco J-810 spectropolarimeter was utilized for all experiments. Temperatures were maintained using a Jasco Peltier controller attachment (PFD-425S). N$_2$ gas flow was set to 100 mL/min. Each trial was the result of co-adding 3 spectra to improve signal:noise and was repeated in triplicate. Spectra were collected from 300 nm to 180 nm in continuous scanning mode (data pitch, 0.1 nm; scanning speed, 50 nm/min; response, 4 sec; bandwidth, 1 nm; cell
width, 1 mm). Spectra were taken from 25 °C to 105 °C in 5 °C increments. Intensity (mdeg) at 222 nm was plotted as a function of temperature to generate melting curves; solution turbidity (HT voltage) at 285 nm was also plotted as a function of temperature to show RecA aggregation.44

Difference FTIR

Both DTT and [1-(2-nitrophenyl)ethyl]adenosine 5’-diphosphate (NPE-ADP, or caged ADP, Invitrogen) were added to RecA aliquots containing approximately 2-3 nmol of protein in a 1:20:200 ratio for protein:caged-ADP:reductant respectively for a total volume of approximately 80 µL in each sample. Samples were partially dehydrated on ice on CaF₂ windows (WFD-424, 19 mm × 2 mm, Harrick Scientific Products) under N₂ flow for approximately 1 hr. Spectra were collected after holding the sample for 4 hrs at -4 °C in a Thermo Nicolet 6700 FT-IR with an MCT/A detector by co-adding 500 scans (optical velocity, 1.8988 cm/s; apodization, Happ-Genzel; resolution, 4 cm⁻¹; phase correction, Mertz).

Caged nucleotide photolysis was accomplished using a nitrogen laser (337 nm) cartridge from Laser Science, Inc. via irradiation for 10 min and taking spectra immediately as well as every 10 min following photolysis for 1 hr. Difference spectra were obtained by taking a ratio of a sample spectrum after photolysis to that before photolysis. This procedure was repeated using samples without protein to subtract out buffer absorbance. Spectra were normalized for nucleotide release using strong 1525 and 1342 cm⁻¹ vibrations corresponding to the loss of NO₂ vibrational modes upon photolytic cleavage, resulting in ΔAbs ~ 0.01. Double-difference spectra were obtained by subtracting spectra without protein from those with protein after normalization for protein content using amide II absorbance intensities since water O-H bending vibrations interfere in the amide I region.
The buffer-specific denaturation profile of RecA was monitored using CD. Loss of signal intensity at 222 nm, indicative of α-helical character, was monitored as a function of temperature to determine the temperature at which RecA unfolds (Figure 6A). Sharp decreases in intensity correspond to protein denaturation. Melting temperatures in each dilute buffer are as follows: Tris, 60 °C; MES, 85 °C; HEPES, 80 °C; Phosphate, >105 °C.

Solution turbidity of each sample was concurrently monitored (Figure 6B). High tension (HT) voltage at 285 nm has been shown to correlate with increased sample turbidity and thus increased aggregation. As the protein partially denatures, RecA species will nonspecifically aggregate. This nonspecific aggregation increases solution
turbidity until the aggregate becomes so large it precipitates from solution and results in a decrease in turbidity (Figure 6B). Monitoring HT voltage serves as a complementary method for determining denaturation temperatures derived from CD as well as observing different aggregation states at 25 °C.

From ~35-45 °C, RecA in all buffers undergoes an initial minor unfolding event. Notably, RecA in HEPES buffer is stabilized in a higher order baseline oligomerization state relative to RecA in Tris, MES, and Phosphate buffers. Following denaturation in MES buffer, turbidity does not return to baseline values yet converges to values similar to HEPES. Phosphate buffer significantly stabilizes RecA compared to all other buffers (Figure 6B).

**Table 2.** Buffer-specific RecA ATPase activities.

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<th>Buffer</th>
<th>Activity (mol ATP/min/mol RecA)</th>
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<tr>
<td>Tris</td>
<td>13.4 ± 0.6</td>
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<tr>
<td>MES</td>
<td>14.2 ± 1.8</td>
</tr>
<tr>
<td>HEPES</td>
<td>10.2 ± 1.9</td>
</tr>
<tr>
<td>Phosphate</td>
<td>11.8 ± 0.3</td>
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To assess buffer-dependent differences in RecA function, ATPase activity was monitored in the presence of ssDNA using a coupled pyruvate kinase-lactate dehydrogenase assay in which 1 mol of ATP hydrolyzed by RecA corresponds to the oxidation of 1 mol of NADH (Figure 4). Buffer-specific activities representing maximal ATPase velocity derived from these assays over the entire time course of the experiments are presented in Table 2. Overall activities are similar in each buffer, yet RecA activity in HEPES and Phosphate buffers may be slightly decreased compared to Tris and MES buffers.
CD spectra of RecA in each buffer were obtained to probe secondary structural differences in each buffer. Spectra presented in Figure 7 show conservation of RecA secondary structure, as shown by characteristic double minima at 208 and 222 nm in all buffers. Only small variations in signal intensity are observed, attributable to slight differences in RecA concentration.

ATR FTIR was also employed to evaluate possible secondary structural differences in each buffer (Figure 8). Spectra are expanded to the amide I and II regions. Amide I (~1650 cm⁻¹ region) primarily arises from carbonyl C=O stretching in the polypeptide backbone whereas amide II (~1550 cm⁻¹ region) is predominantly characterized by amide N-H bending and C-N stretching in the backbone. Spectra were normalized using amide II intensities since water O-H vibrations are not present in the region. No significant shifts or unique spectral features are observed among the spectra, which all exhibit peaks at 1647, 1636, and 1549 cm⁻¹. A modestly
higher intensity 1636 cm\(^{-1}\) peak for RecA in Tris buffer is likely an artifact of the spectra subtraction process.

Difference IR was used to probe unique buffer-specific interactions that influence nucleotide binding and aggregation of RecA. Absorbance spectra were used to normalize protein content between samples using amide II intensities, and background spectral features were determined to be <0.001 ΔAbs by reprocessing two spectra of the same pre-photolytic sample for all trials. Laser-induced cleavage of caged-ADP allows for the determination of conformational changes of RecA upon nucleotide binding. Figure 9 depicts the subtraction scheme for generating difference spectra. A spectrum is taken of RecA+caged-ADP, and following photolysis a spectrum is taken of RecA-ADP+free cage. This experiment is then repeated in the absence of RecA. Each spectrum taken after photolysis (Figure 9, left) is reprocessed against its respective spectrum taken before photolysis (Figure 9, right), yielding a spectrum in the presence and absence of RecA (Figure 10).
Positive peaks correspond to vibrational features present in each sample after photolysis, whereas negative peaks correspond to vibrations that disappear upon photolysis and nucleotide binding to RecA. The positive vibration at 1638 cm⁻¹ is due to the formation of the free cage C=O relative to the pre-photolytic nucleotide structure (Figures 5 and 10, red). Strong negative peaks at 1525 and 1344 cm⁻¹ correspond to the disappearance of the symmetric and asymmetric stretches of the free cage NO₂ functional group, and are used to normalize for the laser-induced release of ADP between samples (Figure 5). Photolytic cleavage of ADP from the cage in the presence of protein yields a similar spectrum with slight differences in the 1700-1600 cm⁻¹ region (Figure 10, blue). Positive vibrational features at 1659, 1645, and 1631 cm⁻¹ are unique to the RecA-ADP bound state with overlapping contributions from the carbonyl of the free cage.

**Figure 9.** Double-difference spectra generation. Spectra taken before (top two, right) photolysis are subtracted from those taken after (top two, left) yielding difference spectra obtained in the presence (top row) and absence (second row) of RecA. These spectra are normalized for photolytic release and ADP-only spectra are subtracted from RecA-ADP spectra. Resulting double-difference spectra reflect binding-induced changes in RecA or nucleotide structure.
Subtraction of these two spectra, or RecA-ADP minus RecA, results in a spectrum solely reflecting binding-induced conformational changes in RecA and nucleotide structure in all buffers (Figure 11). Positive vibrations are correlated with the inactive RecA ADP-bound state. Negative features are associated with vibrations that have disappeared or shifted in the spectrum upon nucleotide binding to RecA. Therefore, these difference IR spectra reflect changes that result from specific ADP binding-induced conformational changes in protein/nucleotide structure. All double-difference spectra in Figure 11 show similar positive vibrational features around 1518-1520 and 1545-1555 cm\(^{-1}\). Phosphate, MES, and Tris buffers all share similar features at 1620 (shoulder), 1622, and 1626 cm\(^{-1}\) and at 1651, 1645, and 1647 cm\(^{-1}\) respectively. Peaks specific to each buffer are annotated in Figure 11. The RecA-ADP minus RecA double-difference spectrum in Phosphate buffer displays unique features at 1685 and 1608 cm\(^{-1}\). MES spectra show an additional positive 1696 cm\(^{-1}\) peak, similar to a positive peak at 1695 cm\(^{-1}\) in
Tris buffer. The HEPES double-difference spectrum shows a negative feature at 1691 cm$^{-1}$ that may correspond to a similar shift in Phosphate buffer, characteristic of the ADP-unbound state. Peaks at 1665 and 1641 cm$^{-1}$ are also unique to the HEPES spectrum.

**Figure 11.** Double-difference spectra of RecA in each buffer with notable vibrations annotated. Each spectrum is the average of triplicate experiments. Tick marks correspond to $4 \times 10^{-3}$ absorbance units.
Discussion

CD unfolding studies in dilute buffers show a significant variability in RecA denaturation temperature (Figure 6). A minor denaturation event occurring at ~35-45 °C in all buffers corresponds to loss of α-helical character as it transitions from its active state structure to a predominantly β-sheet fold as shown previously in our lab. CD and ATR FTIR were used to gain an understanding of the various factors that cause this thermal stability difference. The mechanism could involve numerous factors, including differential buffer-protein stabilizing interactions, buffer-specific alteration of protein conformation, and unique buffer-dependent influences on RecA oligomerization. However, CD and ATR FTIR spectra confirm that RecA secondary structure is conserved and similar in all buffers (Figures 7 and 8). RecA in HEPES buffer exhibits a higher baseline turbidity compared to all other buffers (Figure 6B). Observation of ATPase activity in each buffer shows RecA may be slightly less active in HEPES, which suggests HEPES selectively alters the RecA aggregation state in a manner that is not as conducive for ATP hydrolysis (Table 2).

The spectra presented in Figure 11 represent double-difference FTIR spectra of RecA in an inactive RecA conformation, reflecting buffer-specific changes upon ADP binding in the absence of DNA. Previous double-difference spectra reflecting ADP binding in Tris and HEPES buffers are in good agreement with those shown in Figure 11. Peak intensities of spectra in Figure 11 are on the order of approximately 0.001-0.004 absorbance units, two orders of magnitude lower than amide I intensities in absorbance spectra. These small changes therefore correlate with changes in the protein on a single amino acid level. Secondary structural changes, as well as structural perturbations in the nucleotide itself, may also contribute to the spectra.

Vibrations around 1518-1520 cm⁻¹ in all spectra arise from contributions of nucleotide
vibrations and from RecA-ADP \textit{minus} RecA subtraction artifacts arising from subtracting NO$_2$ stretch vibrations and do not reflect actual binding-induced vibrational differences between the bound and unbound state (Figure 10).\textsuperscript{48} However, certain side chains lining the RecA nucleotide binding pocket are expected to be perturbed upon binding, including glutamine, glutamate, aspartate, asparagine, tyrosine, and lysine residues. Characteristic vibrations of amino acid side chains, as well as structurally-related vibrations, are given in Table 1. Double-difference spectra in Figure 11 show spectral features corresponding to these regions of interest that result from ADP binding.

Changes in RecA secondary structure would be observed in the amide I (1620-1690 cm$^{-1}$) and II (\~1550 cm$^{-1}$) vibrations that indicate protein backbone rearrangements.\textsuperscript{31,46} Qualitatively, RecA is a primarily $\alpha$-helical protein, suggesting amide I features will be located from 1648-1660 cm$^{-1}$\textsuperscript{31} Therefore, positive peaks at 1651, 1645, and 1647 cm$^{-1}$ in Phosphate, MES, and Tris spectra indicate a secondary structural transition upon ADP binding that is not observed in HEPES buffer. In fact, no significant structural changes are observed in the HEPES spectrum as evidenced by the lack of an amide I peak, a modest feature at 1555 cm$^{-1}$ corresponding to amide II, and overall less nucleotide binding-induced changes. The MES spectrum also exhibits minimal amide II changes at 1554 cm$^{-1}$. The Tris double-difference spectrum shows the greatest change upon ADP binding in the 1551 cm$^{-1}$ region. Difference spectra obtained in Phosphate buffer shows a possible 1545 cm$^{-1}$ vibration that overlaps with the 1518 cm$^{-1}$ subtraction artifact. Adenine vibrations present in the nucleotide structure may also contribute to this region (1640-1651 cm$^{-1}$).\textsuperscript{48}

Positive features at 1620, 1622, and 1626 cm$^{-1}$ in Phosphate, MES, and Tris spectra can be linked to aggregation, or formation of RecA filaments, induced by nucleotide binding.\textsuperscript{31}
Importantly, no such feature is evident in the HEPES spectrum. Taken in combination with increased aggregation and modestly lower activity compared to other buffers, this suggests HEPES buffer may promote RecA to a higher order oligomeric state independent of ADP binding (Figure 6B, Table 2). RecA in the ADP-bound and -unbound state is therefore in a similar aggregation state and no significant changes are observed in the double-difference spectrum upon nucleotide binding. This finding is in agreement with previous studies in the lab in which RecA was observed to have reduced yet significant activity in the absence of DNA in 100× HEPES buffer.49 We propose that HEPES buffer therefore acts similar to high salt concentrations in inducing an extended RecA conformation.20,39 In turn, this extended conformation may imitate the active conformation of RecA bound to DNA.2 This unique aggregation state would compete with canonical DNA-dependent ATPase activity, slightly depressing activity, and could explain increased thermal stability in HEPES buffer as compared to Tris. Further activity assays are necessary to exclude HEPES competition with nucleotide binding as a possible explanation for small binding-induced changes in the HEPES double-difference spectrum.

In a similar manner, modestly depressed activity in Phosphate buffer may be ascribed to unique induction and stabilization of the RecA oligomer (Table 2). Phosphates have previously been shown to affect enzymatic activity via direct protein interactions and altering binding affinity.24–26 In this case, phosphate molecules in the buffer may mimic the DNA phosphodiester backbone, screening out efficient DNA binding and affecting RecA activity. This stabilization also likely explains extreme thermal stability of RecA in Phosphate buffer.

MES buffer exhibits similar activity and ADP binding-induced changes in the double-difference spectrum, yet a unique transition between aggregation state upon denaturation
compared to all other buffers. However, further studies are necessary since it is unclear how MES buffer stabilizes RecA compared to Tris. In all buffers, altered secondary structure,\textsuperscript{46} protein aggregation,\textsuperscript{37,45} and nucleotide\textsuperscript{48} vibrations compete with numerous side chain stretches\textsuperscript{31,38,42} throughout the spectral region shown in Figure 11. These vibrations, listed in Table 1, may give rise to peaks in the double-difference spectra and overlap with assigned vibrations. Therefore, further investigation is warranted to disambiguate these assignments.
Conclusions

In conclusion, the cause for RecA thermal stability differences in each of four common buffers was evaluated using various spectroscopic and biochemical techniques. To explain this phenomenon, CD and ATR FTIR were used to show that global RecA secondary structure is conserved among all four buffers. Assays showed similar ATPase activity in each buffer, yet RecA in HEPES and Phosphate buffers may exhibit slightly less activity compared to Tris and MES. Difference FTIR spectroscopy used in conjunction with laser-induced photolysis of caged nucleotides reveals interesting differences in ADP binding to RecA in each buffer. RecA-ADP minus RecA double-difference spectra obtained in Tris and MES buffers show very similar vibrational changes upon nucleotide binding. The mechanism by which MES buffer stabilizes RecA remains to be elucidated. However, RecA-ADP minus RecA spectra obtained in Phosphate and HEPES buffers exhibit interesting features. Phosphate buffer is inherently unique in that its structure mimics that of the phosphodiester backbone of DNA to which RecA binds. Buffer screening of the DNA-binding site on RecA may induce a specific RecA oligomer that endows extreme thermal stability to the protein. Furthermore, RecA in HEPES buffer is promoted to a unique aggregation state that may mimic the extended conformation of RecA in high salt concentrations. This aggregate is able to imitate RecA bound to DNA, slightly decreasing DNA-dependent ATPase activity. Further work is planned to more clearly assign double-difference spectra and establish a direct link between buffer identity and RecA stabilization.
References


