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Antagonistic effects Na^+ and Mg^{2+} on the structure, function, and stability of mycobacteriophage L1 repressor

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Temperate mycobacteriophage L1 encodes an unusual repressor (CI) for regulating its lytic-lysogenic switching and, in contrast to the repressors of most temperate phages, it binds to multiple asymmetric operator DNAs. Here, ions like Na^+, Cl^-, and acetate$^-$ ions were demonstrated to facilitate the optimal binding of CI to cognate operator DNA, whereas K^+, Li^+, NH_4^+, Mg^{2+}, carbonate^{2-}, and citrate^{3-} ions significantly affected its operator binding activity. Of these ions, Mg^{2+} unfolded CI most severely at room temperature and, compared to Mg^{2+}, Na^+ provided improved thermal stability to CI. Furthermore, the intrinsic tryptophan fluorescence of CI was changed notably upon replacing Na^+ with Mg^{2+} and these opposing effects of Mg^{2+} and Na^+ were also noticed in their actions on the C-terminal fragment (CTD) of CI. Taken together, Na^+ appeared to be more appropriate than Mg^{2+} for maintaining the biologically active conformation of CI needed for its optimal binding to operator DNA. [BMB reports 2009; 42(5): 293-298]

INTRODUCTION

The repressor proteins, expressed by temperate mycobacteriophages L5 (1, 2), Bxb1 (3) and L1 (4) have been reported to bind to multiple asymmetric operator DNA sites and L5 operators, in particular, have been shown not only to repress transcription from its early promoters (5) but also to stop the elongation of L5 transcripts (2). Interestingly, L1 repressor (CI) and its cognate operator DNAs are 100% sequentially identical to L5 (4). An L1 CI variant carrying the P131L change binds to operator DNA at 32°C but not at 42°C (6), however another L1 CI variant devoid of its putative helix-turn-helix (HTH) motif did not bind to cognate operator DNA at 32-42°C (7). L1 CI is a 183 residue polypeptide with a small hinge region flanked by an N-terminal domain (NTD) and a C-terminal domain (CTD). Both CTD and CI form dimers in solution, have a substantial amount of α-helix at 30°C, and unfold at higher temperatures (8). L1 CI binds to different operator DNAs with variable affinity in a temperature sensitive manner.

Temperature, pH, salts, and ions have considerable affect on the operator DNA binding affinities of various repressors (9-12). L1/L5 repressor was reported to bind to the cognate operator DNA at 0°C in the presence of 45-50 mM NaCl (2, 6), and at NaCl concentrations greater than 50 mM CI binding activity was substantially reduced. Notably, 50 mM solutions of NH_4Cl, KCl, LiCl, and Na_3-citrate also significantly affected L1 CI's binding affinity (7). Thus far, no systematic work has examined the effects of different ions on the structure-function relationship of any mycobacteriophage repressor. Here the effects of eight widely used salts on mycobacteriophage L1 repressor were studied using various *in vitro* techniques. For the first time Na^+ and Cl^- or acetate$^-$ were demonstrated to appreciably stabilize the structure of the repressor and were required for the optimal binding of L1 repressor to cognate operator DNA, while Mg^{2+} not only unfolded and destabilized the L1 repressor but significantly affected its function.

RESULTS AND DISCUSSION

Na^+ maintained the biologically active conformation of L1 repressor

Previously it was reported that 50 mM NaCl is required for the optimal binding of L1 repressor to its cognate operator DNA (7), however, as the L1 repressor used then was semipurified and no systematic equilibrium binding study was included, the above data may not be conclusive. Recently, CI was purified to homogeneity and characterized to some extent (8). The requirement of Na^+ for maintaining the biologically active conformation of CI was investigated by equilibration of CI in NaCl, KCl, LiCl, or NH_4Cl buffer and the structure and function of the CI examined by standard *in vitro* methods and comparison of the results. Fig. 1A-D show the equilibrium binding of CI by gel shift assays carried out in the presence of Na^+, K^+, Li^+, and NH_4^+, respectively. The plots of the percent of bound repressor versus repressor concentration showed that ½-maximal binding of repressor (considered here as the apparent equilibrium dissociation constant, K_D) was distinct for each chloride salt.

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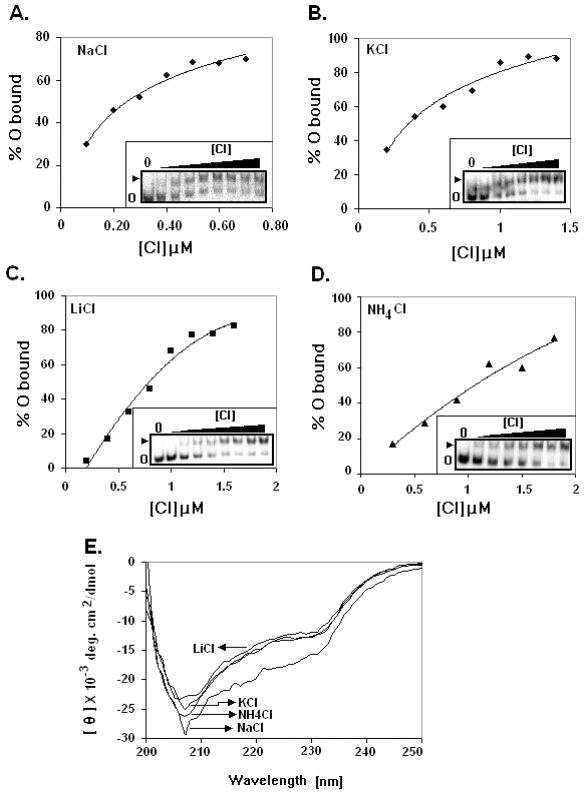

Fig. 1. Effects of monovalent cations on L1 CI. Equilibrium binding of (A) NaCl, (B) KCl, (C) LiCl, and (D) NH_4Cl -equilibrated CI to 0.1 nM ^{32}P labeled *O*64 operator DNA (O) Repressor concentrations are not identical in all assays but kept within 0.1-1.8 μM. Arrowhead indicates the CI-*O* complex. From the autoradiogram of gel shift assay (inset picture), the % operator bound (in the CI-*O* complex) in a specific salt buffer was estimated and plotted against the corresponding CI concentration. (E) CD-spectra (200-260 nm) of 15 μM CI in NaCl, KCl, LiCl, and NH_4Cl buffers. To exclude noise, an average of 3 scans in each buffer presented here.

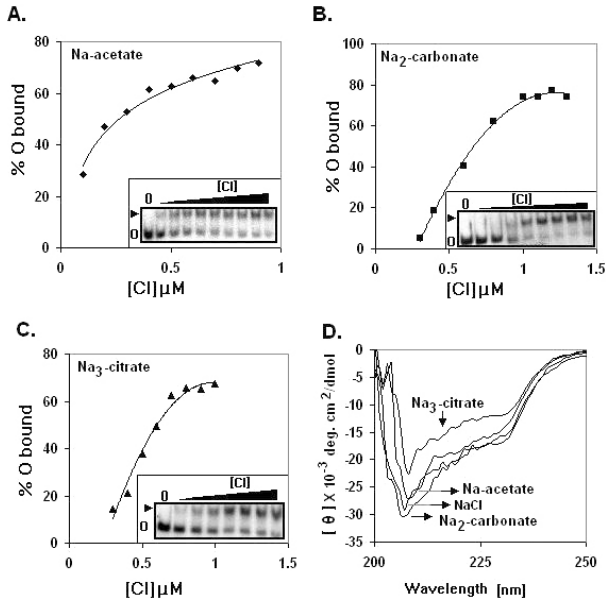

Fig. 2. Effects of anions on L1 CI. Equilibrium binding of (A) Na-acetate, (B) Na_2-carbonate, and (C) Na_3-citrate-equilibrated CI to 0.1 nM ^{32}P labeled O_{64} operator DNA (*O*). (D) CD-spectra (200-260 nm, 3 scan average) of 15 μM CI in the presence of indicated salts.

The K_D values for the repressor-operator interaction in Na^+, K^+, Li^+, and NH_4^+ buffers were calculated to be 250, 350, 800, and 1065 nM, respectively, and indicated that the affinity of the repressor for the operator DNA in NaCl buffer was about 1.4-4-fold stronger than in the other three buffers. Na^+ was therefore better than K^+, Li^+, and NH_4^+ for producing the optimal binding of the repressor.

The possibility that stabilization of the proper biological conformation of CI by Na^+ at room temperature may in turn lead to stronger binding of CI to its cognate operator DNA was tested by collecting CD spectra (200-260 nm) of the salt-equilibrated CI solutions (Fig. 1E). The spectrum of CI in the presence of NaCl showed a peak of large negative ellipticity at 208 nm and analysis of this spectrum by CDNN (13) revealed that the CI was about 23.5% α-helical, 20% β-stranded, and 39% coiled in structure. CD spectra of CI pre-equilibrated in KCl, LiCl, or NH_4Cl buffer also exhibited strong peaks at 208 nm, but the CDNN analysis showed about a 6-12% reduction of α-helical structure when NaCl was replaced by another monovalent cation chloride salt. Taken together, the data suggested that none of these cations significantly unfolded CI.

As cations interact with the negatively charged moieties of both protein and DNA, NH_4^+, K^+, and Li^+ ions appeared to maintain or at least not significantly disturb, as indicated above, the conformation of CI in a manner similar to Na^+, but differ from Na^+ in their affect on its operator binding activity. These three cations possibly masked the negative charges of the operator DNA, inhibiting the binding of CI. The slight structural modification in CI induced in the presence of these ions may also partly contribute to CI's reduced operator binding affinity. In this respect, the superior effect of Na^+ in stabilizing the conformation and in allowing optimal binding of CI to operator DNA was quite unexpected, but it was not clear which effect or if both effects were required for the observed binding maximization. Most possibly, the effective size of the Na^+ ion was more appropriate here than NH_4^+, K^+, and Li^+ for maintaining the proper conformation of CI and forming the stable complex between CI and the operator DNA.

Effects of acetate$^-$, carbonate^{2-}, and citrate^{3-} on L1 repressor

The effects of different anions on the function of L1 repressor was examined by equilibrating CI in Na-acetate, Na_2-carbonate, or Na_3-citrate buffer and the binding of CI measured by gel shift assays (Fig. 2A-C) and the resulting data compared with that obtained using 50 mM NaCl (Fig. 1A). The K_D values for the repressor-operator

interaction in Na-acetate, Na_2-carbonate, and Na_3-citrate buffers were estimated to be 275, 680, and 600 nM, respectively, indicating that Na^+ and Cl^- or acetate$^-$ ions were crucial for the optimal binding of the L1 repressor to operator DNA.

CD spectra (200-260 nm) of the above sodium salt-equilibrated CI solutions revealed that the spectrum of CI in the Na-acetate buffer was nearly identical to that from the NaCl buffer (Fig. 1E), whereas the spectra of CI recorded in Na_3-citrate and Na_2-carbonate buffers were slightly different from the others (Fig. 2D). These results suggested that none of the anions unfolded CI severely, but small conformational changes of CI might have occurred in the Na_2-carbonate and Na_3-citrate buffers.

As anions can interact with the positively charged amino acid residues of a protein, Cl^-, acetate$^-$, citrate^{3-}, and carbonate^{2-} ions could have interacted with the Lys and Arg residues of CI and caused distinct CI conformational changes. Apparently here, the conformation of CI, as maintained by Cl^- and acetate$^-$, was suitable for its optimal binding, but minor conformational changes in CI in the presence of carbonate^{2-} or citrate^{3-} was possibly not suitable for optimal binding of CI to the operator DNA.

Mg^{2+} severely affected the structure, function, and stability of L1 repressor

The effect of Mg^{2+} on the function of L1 CI was studied by examination of the equilibrium binding of CI in 50 mM $MgCl_2$ buffer through gel shift assay (Fig. 3A). The K_D value, determined from the plot of percent of repressor bound versus repressor concentration, was about 675 nM, which suggested that the affinity of repressor for the operator DNA in $MgCl_2$ buffer was about 2.7-fold weaker than was observed in equimolar NaCl (Fig. 1A).

The CD spectra of $MgCl_2$-equilibrated CI displayed a much reduced peak at 208 nm in comparison with NaCl buffer (Fig. 3B), representing a 44% reduction of α-helical and concomitant 15% increase of random coil structure in the CI relative to its structure in NaCl. Confirmation that Mg^{2+} really affects the conformation of CI was obtained through CD spectra of CI pre-equilibrated separately in 100 mM NaCl or in NaCl and $MgCl_2$ (50 mM each) buffer. The results revealed that the CD spectrum of CI in the NaCl and $MgCl_2$ buffer was very similar to that seen in 50 mM $MgCl_2$ buffer alone, but the negative ellipticity of the peak of both spectra was about 30% less than that of CI in the 100 mM NaCl buffer at 208 nm. Further analysis indicated there was a 21.5% decrease of α-helical and a concomitant 8% increase of random coil structure in CI when NaCl buffer was combined 50 mM with $MgCl_2$; in other words, there was roughly half the effect that was observed with 50 mM $MgCl_2$ buffer alone. Taken together, the data indicate that Mg^{2+} destabilized as well as unfolded CI severely which most probably led to poorer binding of CI to operator DNA. In addition, these results indicated that increasing NaCl concentration also unfolded CI substantially.

Considering the above results, it appeared that if Na^+, at appropriate concentrations, stabilized CI structure (as evident from above analysis), then aggregation of CI at elevated temperatures should be resisted more in a NaCl buffer than a $MgCl_2$ buffer. Thus, the thermal aggregation of CI was examined by the standard procedure (Fig. 3C) and it was found that aggregation of NaCl-equilibrated CI was initiated at ~34°C, increased rapidly with increasing temperature, and finally reached saturation at ~43°C. In contrast, the aggregation pattern of $MgCl_2$-equilibrated CI was almost the same except that aggregation was initiated at ~29°C, clearly indicating that Na^+ indeed stabilized CI more than Mg^{2+}.

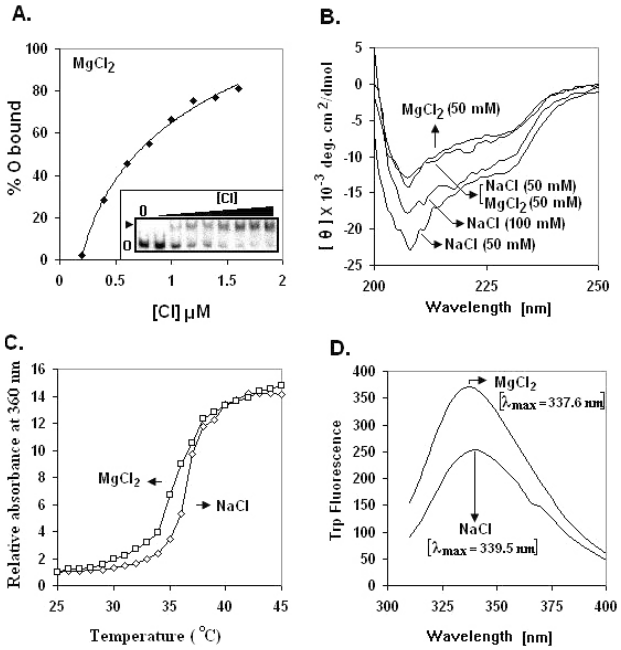

Fig. 3. Effect of Mg^{2+} on L1 repressor. (A) Equilibrium binding of 50 mM $MgCl_2$- equilibrated CI to 0.1 nM ^{32}P labeled O_{64} operator DNA (O). (B) CD-spectra (200-260 nm) of 10 μM CI in the presence of indicated salts. (C) Thermal aggregation of 5 μM CI in NaCl or $MgCl_2$ buffer, each preformed in triplicate, one result shown here. (D) Intrinsic tryptophan fluorescence of 5 μM CI pre-equilibrated in NaCl or $MgCl_2$ buffer and measured at room temperature, each performed in quintuplicate, one scan shown here.

L1 repressor contains seven tryptophan residues at positions 50, 69, 71, 112, 113, 170, and 172 (4). The effect of Na^+ and Mg^{2+} on the tryptophan environment of CI as examined by recording the intrinsic tryptophan fluorescence spectra of CI in the presence of NaCl and $MgCl_2$ (Fig. 3D) and showed that the fluorescence spectrum of NaCl- equilibrated CI displayed a red-shifted emission maximum at 339.5 nm, whereas the spectrum in $MgCl_2$ buffer showed a maximum at 337.6 nm. As the emission maximum of a protein with buried tryptophans generally lies at 335 nm or less (14) and usually changes with changes in the conformation of a protein (15), this data indicated that tryptophan residues of CI in NaCl buffer were more exposed than in MgCl2 buffer and that the conformation

of CI in these two buffers differed significantly.

Curiously, the tryptophan fluorescence intensity of CI in the NaCl buffer was less than in the $MgCl_2$ buffer (Fig. 3D). Although the reason for this unexpected phenomenon is not known with certainty, one possibility is that L1 repressor molecules might have formed microaggregates in the $MgCl_2$ buffer, causing the increased fluorescent intensity.

In comparison with monovalent cations, divalent Mg^{2+} might compete more strongly with the repressor for binding to the operator DNA and in turn lead to a massive charge neutralization of the DNA and, as a result, repressor protein cannot bind to operator DNA unless present at sufficiently higher concentrations. Alternatively, the reduced operator binding affinity of L1 CI in $MgCl_2$ buffer might be due to both charge neutralization of operator DNA and concurrent crippling of its structure.

Opposing actions of Na^+ and Mg^{2+} on the conformation and stability of CTD

The effects of Na^+ and Mg^{2+} on the C-terminal domain (CTD) of CI were also investigated by several *in vitro* experiments using CTD in place of CI (Fig. 4A). The CD spectrum (200-260 nm) of NaCl-equilibrated CTD contained a peak of large negative ellipticity at 208 nm, while the corresponding peak from the CTD spectrum in $MgCl_2$ buffer was substantially reduced . As expected, a significant reduction of α-helical and an increase of random coil structure in CTD occurred when NaCl was replaced with $MgCl_2$, indicating that Na^+ was superior to Mg^{2+} at maintaining the conformation of CTD, the most stable region of CI (8).

Again in parallel to studies of CI, thermal aggregation studies of CTD revealed that aggregation of CTD pre-equilibrated in NaCl and $MgCl_2$ buffers started at ~39° and ~31°C, respectively (Fig. 4B) and, after the initiation of aggregation, both events proceeded rapidly with increasing temperature. As previously observed with CI, these results suggested that Na^+ provided more thermal stabilization to CTD than Mg^{2+} and, also curiously, the CTD aggregation initiated at higher temperatures than did CI in both of these buffers. Uncertain as to exactly why this occurred, one possibility is that CTD without the NTD region of CI attained a more stable conformation than could intact CI.

The CTD of L1 repressor, which carries six Trp residues, has been reported to be resistant to chymotrypsin digestion (8), indicating that all the six Trp residues might be buried in the folded CTD interior and that the intrinsic fluorescence from such inaccessible Trp residues should display a blue-shifted emission maximum. Tryptophan fluorescence spectrum of CTD in NaCl buffer, in fact, showed an emission maximum at 335.6 nm (Fig. 4C), while the emission maximum of CTD in $MgCl_2$ buffer was observed at 336.8 nm. With the added observation that the fluorescence intensity of CTD in the latter salt was also considerably reduced, these results suggested that the Trp environment as well as the conformation of CTD in the NaCl buffer was different from in the $MgCl_2$ buffer.

The effects of monovalent and polyvalent ions on the DNA binding activity of L1 CI (discussed above) was only partly similar to those that have been reported for other repressors. $Acetate^-$, Cl^-, and $citrate^{3-}$ have been shown to affect the DNA binding activity of LacI profoundly, whereas Li^+ was found to be better than Na^+ or K^+ for the optimal DNA binding of LacI (10), and the DNA binding activity of LexA was unaffected upon replacing Na^+ with K^+ (12). *E. coli* trp repressor that was neither stabilized nor affected by Na^+ and K^+ needs $(NH_4)_2SO_4$ for optimal DNA binding (17). Interestingly, both K^+ and Ca^{2+} were required for the optimal binding of λ CI repressor to cognate operator DNA (20). Even Mg^{2+} was shown to significantly enhance the affinity of λ CI to its operator DNA, but Mg^{2+} exhibited a severe destructive effect on the DNA binding activity of Lac1 (10, 16), LexA (12), trp (17), and AraC (21) repressor proteins.

MATERIALS AND METHODS

E. coli strain and salts

E. coli cells were routinely grown in Luria-Bertani (18) me-

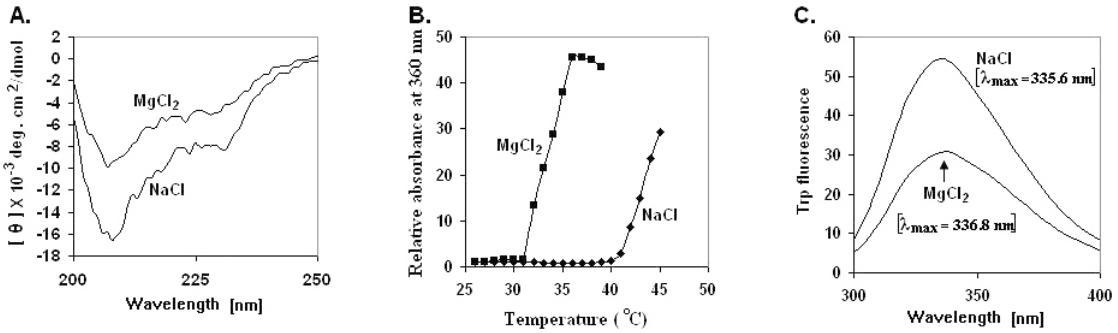

Fig. 4. Effects Na^+ and Mg^{2+} on CTD of CI. (A) CD-spectra, (B) thermal aggregation, and (C) intrinsic tryptophan fluorescence of 10 μM CTD pre-equilibrated in NaCl or $MgCl_2$ buffer.

dium (supplemented with appropriate antibiotics as needed). All chemicals including various salts were purchased from Sigma, USA and EMarck, Germany.

Molecular biological techniques

Agarose gel electrophoresis, DNA estimation, restriction enzyme DNA digestion, protein estimation, native and SDS-PAGE, staining of gel, and labeling of O_{64} operator DNA with either [^{32}P-γ] ATP or [^{32}P-α] dATP (BARC, India) etc. were carried out by standard procedures (8, 18, 19, 22).

Purification and estimation of proteins

Purification of histidine-tagged L1 repressor (CI) was described previously (8). The C-terminal domain of L1 repressor (CTD) was purified by partial digestion of repressor with chymotrypsin followed by the purification of CTD as previously described for purification of trypsin-generated CTD (8). The concentration of CI/CTD was calculated using the molecular mass of monomeric CI/CTD.

Gel shift assay

The equilibrium binding of L1 CI to 0.1 nM O_{64} operator DNA (120 bp) in NaCl buffer (20 mM Na-phosphate buffer, pH 6.0, 50 mM NaCl, 1 mM EDTA, and 5% glycerol) was measured using a standard gel shift assay (6, 8) and band intensities in the autoradiograms determined by the previously published procedure (8).

The effects of different ions on L1 CI-operator interactions were examined by the preparation of NaCl buffers in which the NaCl was replaced by an equimolar concentration of $MgCl_2$, NH_4Cl, KCl, LiCl, Na_3-citrate, Na-acetate, or Na_2-carbonate. L1 repressor in NaCl buffer was dialyzed extensively against each modified buffer at 4°C and, as the repressor was not precipitated in any of the these buffers, they were utilized in the experiments performed here.

CD spectra

Circular Dichroism (CD) spectra (200-260 nm) of 10-15 μM repressor, pre-equilibrated in NaCl buffer or in modified buffers, were recorded by JASCO J600 spectrophotometer at 25°C as well as spectra of 10 μM CTD equilibrated in NaCl and $MgCl_2$ buffers. Samples were transferred to a 1 mm path length cuvette prior to the recording of CD spectrum.

Thermal aggregation

Temperature-induced aggregation of 5 μM CI/10 μM CTD in NaCl buffer or in $MgCl_2$ buffer measured by monitoring the absorbance at 360 nm in a spectrophotometer (Shimadzu 3000) connected to a temperature-regulated water bath such that the cuvette was heated to a desired temperature, equilibrated for 3 min, and the absorbance recorded.

Tryptophan fluorescence spectra

Intrinsic tryptophan fluorescence spectra of 5 μM CI/10 μM CTD in NaCl buffer or in $MgCl_2$ buffer were recorded with a Hitachi F-3010 spectrofluorimeter with a 5 nm bandpass (for both excitation and emission), excitation at 295 nm, and emission recorded from 300-400 nm.

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