The Charge of Ribonuclease A at pH 6 and 7

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Real-time electrophoretic mobility (REM) measurements in membrane confined electrophoresis (MCE) were used to determine the charge of ribonuclease A (RNase A) in various buffer conditions at pH 6 and 7. This work demonstrates the difference in charge due to the binding of phosphate to RNase A at pH 6 and provides useful insight to further investigate charge conservation within a system.

Introduction

The charge of ribonuclease A (RNase A) and C-domain swapped dimer (C-dimer) were measured using real-time electrophoretic mobility (REM) in membrane confined electrophoresis (MCE) at pH 6 and 7 under various buffer conditions. In this study, the charge of RNase A monomer was measured at pH 6 with and without the presence of phosphate and then compared. Additionally, this work provided useful insight for further investigation into the conservation of charge within a system.

Materials & Methods

Protein and chemicals: Purified lyophilized powder of RNase A type XIIA and C-domain swapped dimer were obtained and used without further purification (courtesy of G. Gotte, University of Verona). The chemicals used included potassium chloride, bis-tris-propane (BTP), and potassium phosphate (monobasic and dibasic). All chemicals were of analytical grade and purchased from Sigma Aldrich.

Sample and membrane preparation: BioTech grade regenerated cellulose 3.5-5k MWCO membranes were used for all experiments (Spectrum Labs). Membranes were pre-soaked in running buffer for a minimum of 10 minutes prior to each experiment. Each sample solution was dialyzed against the run buffer for a minimum of 24 hours using 3.5-5k MWCO dialysis cassettes (Thermo Scientific). The concentration of RNase A was determined spectroscopically at 280 nm using an extinction coefficient of 0.72 g/l.

REM-MCE: Approximately 20 µl of solution containing 0.5 mg/ml of RNase A was loaded into the 2x2x4 mm^3 sample chamber. The electrophoretic mobility of RNase A and the C-dimer were measured at pH 6 and 7 under the following buffer conditions:

- 100 mM KCl, 10 mM $K_2PO_4^{2-}$, pH 7.0
- 100 mM KCl, 10 mM BTP, pH 6.0
- 100 mM KCl, 10 mM BTP, 10 mM $K_2PO_4^{2-}$, pH 6.0
- 100 mM KCl, 10 mM BTP, 50 mM $K_2PO_4^{2-}$, pH 6.0

It should also be noted that because the Stokes radius (R_s) of the C-dimer is unknown, the R_s value used for C-dimer charge calculations was two times the monomer or 3.56 nm.

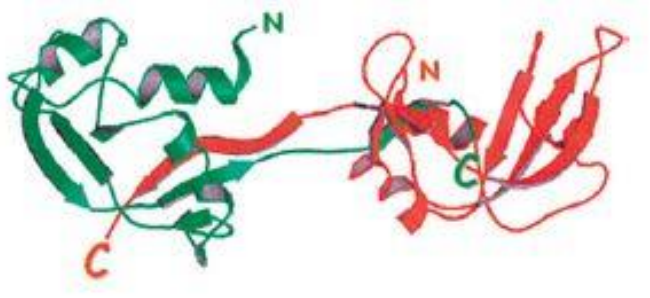

Figure 1 The C-dimer shown at 1.75 Å resolution. The dimer is formed by the swapping of the C domains of both monomers (Libonati, Gotte, 2004).

Results

Table 1 displays the experimentally determined charge of RNase A monomer and the C-dimer in various buffer conditions at pH 6 and 7. For all RNase A experiments at pH 7, convection was observed instead of the typical moving boundary. This behavior suggests that both the monomeric and dimeric forms of RNase A are charge neutral at pH 7 under these buffer conditions.

Table 1 Ribonuclease A type XIIA and C domain swapped dimer charge results

Buffer[a,b]	pH	Sample	Mobility (x 10^{-5} $cm^2/V*s$)	Z_{eff}[c]	Z_{DHH}[d]
100 mM KCl, 10 mM $K_2PO_4^{2-}$	7.0	RNase A	-	NC	NC
		C-dimer	-	NC	NC
100 mM KCl, 10 mM BTP	6.0	RNase A	5.48 ± 0.70	1.15 ±0.15	3.36 ± 0.43
100 mM KCl, 10 mM BTP, 10 mM $K_2PO_4^{2-}$	6.0	RNase A	4.75 ± 0.37	1.0 ± 0.23	3.0 ± 0.24
		C-dimer	6.09 ± 0.94	1.28 ± 0.16, 2.56 ± 0.39	3.85 ± 0.48, 11.86 ± 1.81
100 mM KCl, 10 mM BTP, 50 mM $K_2PO_4^{2-}$	6.0	RNase A	-	NC	NC
		C-dimer	-	NC	NC

[a] **100 mM KCl, 10 mM BTP: Γ = 0.11 mol/L, k = 14.26 mS/cm**
[b] **100 mM KCl, 10 mM BTP, 10 mM $K_2PO_4^{2-}$: Γ = 0.1224 mol/L, k = 14.64 mS/cm**
[c] **Reduced valence calculations used: η = 1.0027, RNase A (monomer) R_s = 1.78 nm[1]**
[d] **Debye-Hückel-Henry calculations used: C-Dimer R_s = 3.56 nm, Chloride (Cl^-) counter-ion R_s = 0.122 nm[1]**
NC = no charge; charge neutral

In Table 1, notice that there are two reported charge values for the C-Dimer in 100 mM KCl, 10 mM BTP, 10 mM K2PO4, pH 6. It was found that the C-dimer data is consistent with the dimer disassociating to monomer, making it difficult to know the size of the macro-ion actually being measured. Thus, the first charge value reported was calculated using the R_s of the monomer, 1.78 nm. The second charge value listed used two times the R_s of the monomer (3.56 nm) as an estimate for the R_s of the C-dimer.

Monomer: Figure 2 displays the difference in the Debye-Hückel-Henry (Z_{DHH}) charge of RNase A at pH 6 with and without the presence of phosphate. A total of 10 REM experiments were conducted under each buffer condition and averaged.

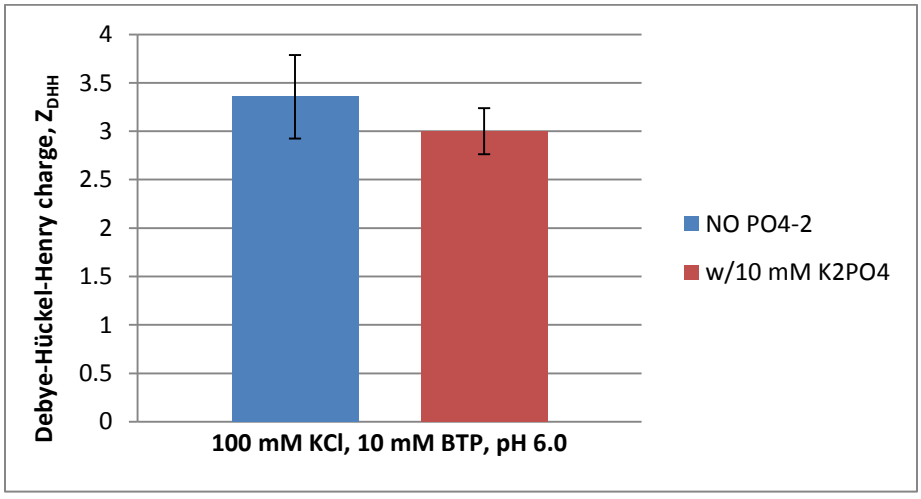

Figure 2 Debye-Hückel-Henry charge (Z_{DHH}) of RNase A type XIIA in 100 mM KCl, 10 mM BTP buffer at pH 6 with (Red) and without (Blue) the presence of 10 mM potassium phosphate.

C-Dimer: The C- dimer was measured only in buffers containing > 5 mM phosphate because a small amount is required for the dimer to remain stable.[2,3] The electrophoretic mobility of the C-dimer was measured in 100 mM KCl, 10 mM BTP, 10 mM $K_2PO_4^{2-}$ at pH 6, repeated three times and averaged. The C-dimer mobility data acquired in all three experiments had a lower signal to noise ratio than typical REM data. It was thought that the data's low signal to noise was due to the C-dimer disassociating as the experiment progressed. In an attempt to help stabilize the molecule and prevent disassociation, the C-dimer was then measured under the same buffer conditions (100 mM KCl, 10 mM BTP pH 6) however, the phosphate concentration was increased from 10 mM to 50 mM. Consequently, the increase in phosphate concentration neutralized the C-dimer, causing the sample to convect.

The monomer was then run under the same conditions to see if it too was neutralized when the phosphate concentration was increased from 10 mM to 50 mM. As a result, the monomer also became charge neutral when the phosphate concentration was increased.

Conclusion

It was experimentally determined that RNase A in both its monomeric and dimeric (C-dimer) form are charge neutral in 100 mM KCl, 10 mM $K_2PO_4^{2-}$ at pH 7.

The charge of RNase A and the C-dimer were measured in 100 mM KCl, 10 mM BTP, 10 mM $K_2PO_4^{2-}$ at pH 6 and compared. The data suggests that the monomer weakly associates into dimer which would account for the noisy data sets as well as the calculated charges being closer to the monomer value (See Table 1). It is important however, to remember that the R_s of the C-dimer was not directly measured for the work described here, and that a more definitive conclusion can be made once the Stokes radius has been measured and a more suitable buffer condition is determined.

It was also found that the charge of the monomer in the presence of phosphate differs only slightly from the charge of the monomer without phosphate present. However, as shown in Figure 1, it appears as though the presence of phosphate improved the precision of the measurement. Additionally, when the phosphate concentration was increased from 10 mM to 50 mM at pH 6 both forms of RNase A became charge neutral.

References

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