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Influence of Arginine Salts on the Thermal Stability and Aggregation Kinetics of Monoclonal Antibody: Dominant Role of Anions

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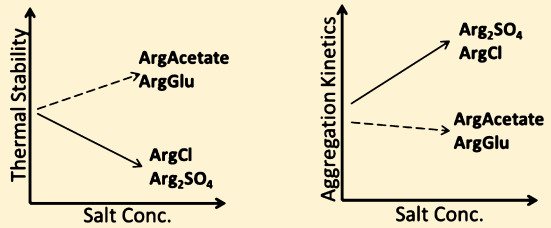
Supporting Information

ABSTRACT: Thermal stability of the C_H2 domain for an IgG1 monoclonal antibody and its aggregation kinetics were systematically studied at pH 4.8, below its pI of 8.8 in individual solutions of arginine salts with acetate, glutamate (Glu^-), chloride, and sulfate as the anion, in comparison to sodium chloride and sodium sulfate. Thermal unfolding temperature, T_m, an indicator of thermal stability, was measured by both differential scanning calorimetry (DSC) and differential scanning fluorimetry (DSF). The aggregation kinetics was determined by assessing reversibility for the C_H2 domain in the DSC repetitive scans and then cross-examined by the isothermal aggregation study measured by size exclusion chromatography. The effect of Arg^+ on the thermal stability and aggregation kinetics of the antibody is shown to be strongly anion-dependent: both ArgAceate and ArgGlu improve the stability, while both Arg_2SO_4 and ArgCl decrease it. Furthermore, the addition of ArgCl and Arg_2SO_4 accelerates the aggregation kinetic, but to a lesser extent than the respective Na^+ salt, suggesting that Arg^+ binds to the antibody more strongly than Na^+. However, the binding of Arg^+ did not lead to more destabilization of the C_H2 domain by the Arg^+ salts at low concentrations, comparing to the respective Na^+ salt. This finding indicates that Arg^+ prefers the protein surface, rather than the exposed backbone upon unfolding. Furthermore, the change in the ranking for affecting the thermal stability and aggregation kinetics as the salt concentration increases implies the presence of other multiple mechanisms, e.g., cluster formation through the homoion pairing between Arg^+ molecules and their preferential exclusion from the protein surface, and heteroion pairing between Arg^+ and SO_4^{2-}.



KEYWORDS: *arginine, antibody, Hofmeister salts, protein−protein interactions, thermal stability, protein aggregation*

INTRODUCTION

In order to manufacture high quality aqueous drug products, it is essential to maintain both short-term and long-term stability of protein solutions during processing and storage. Stability of protein solutions during processing and storage is required to make scalable drug products. Cosolutes (or excipients), e.g., salts, amino acids, and sugar, are routinely added into protein liquid formulation to optimize protein stability. While arginine ion (Arg^+) is one of the widely used cosolutes for protein solubilization and aggregation suppression,[1−3] it is still an active research area of studying Arg^+'s perturbation on the thermodynamics and kinetics aspects on protein solution behavior, such as thermal stability and aggregation.

There are several studies in literature demonstrating the utility of arginine salts. It has been reported that the unique combination of glutamic acid with Arg^+ at 50 mM improves protein solubility and stability more drastically, than the use of 25 mM in combination or either of the amino acids alone.[4] However, it has been reported that Arg^+ can even destabilize proteins[5] and accelerate aggregation kinetics.[6] To understand the inconsistent effect on protein solution behavior by Arg^+, it is important to investigate both the biophysical properties of a protein and the solution conditions from the physicochemical properties of Arg^+ and its companion anions. The delineation of the mechanisms of the interaction between Arg^+ and a protein can help rationalize protein thermal stability and aggregation behavior.

First, the effect of simple salt ions (including both cations and anions) on protein stability and solubility is historically defined by the direct Hofmeister series.[7] For example, the anions typically follow the order of $SCN^- > Cl^- > SO_4^{2-}$ at destabilizing proteins. It has also been shown that the effect of monovalent anion is more pronounced than that of monovalent cation.[8] Importantly, other experimental evidence reveals that the specific interactions between ions and protein surface drive the macroscopic protein solution behavior,[9] such as thermal stability and aggregation.

Second, specific ion effects could be intertwined with the diverse biophysical properties on protein surface, where there are neutral amide peptide backbone, charged side chains, and hydrophobic side chains. It is now well acknowledged that

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monovalent chaotropic anions follow the Law of Matching Water Affinities for their binding strength to protein surface,[10] e.g., the weakly hydrated SCN^- interacts much more strongly than Cl^-, through the positive side chains of Arg^+, Lys^+, and amide backbone on the protein surface. However, the strongly hydrated SO_4^{2-} only strongly interacts with positive-charged side chains.[11] The strongly hydrated cation, e.g. Mg^{2+}, interacts with the negative-charged carboxylates on protein surface, while the weakly hydrated Na^+ interacts to a much less extent.[12]

Third, biophysical properties of a protein can also be tuned by solution conditions, modulating protein aggregation kinetics and thermal stability behavior. Recently, direct and reverse Hofmeister series were discovered for both cations and anions, dependent on the relationship between solution pH and pI of a protein.[13,14] This type of behavior could be correlated to the net charge of a protein. As pH increases from below, through, and above the pI of a protein, the net charge of a protein is positive, neutral, and then negative, respectively. It has been shown experimentally that the greater the positive charge a protein carries, the more intense the modulation of surface hydration is by anions.[15] In addition, proteins are more stable conformationally and thermally at pH conditions near the pI.[16] Specifically, monoclonal antibodies tend to unfold and aggregate irreversibly in acidic pH conditions.[17] Furthermore, for a net positive-charged protein, the extent of the attenuation to repulsive electrostatic protein−protein interactions by anions typically follows the reverse Hofmeister series,[13,14] possibly countered by the interaction of the cation with the protein surface.[13] Thus, anion-binding results in faster protein aggregation, while cation-binding decreases the effectiveness of the anion.[18]

Often the effect of a cosolute, e.g., salt ions, on protein thermal stability is more complex because how the cosolute interacts with both native (N) and unfolded (U) states determines the perturbation on thermal stability.[19] Specifically, a cosolute can decrease the thermal stability through lowering the free energy state of U more than that of N by interacting more favorably with protein surface (preferential binding) because the U state has more solution-exposed surface area than the N state. From a point of view of protein−protein interactions on colloidal stability, the preferential binding should result in repulsive protein−protein interactions and slower aggregation kinetics.[20] However, when hydration of a cosolute is energetically preferred over protein surface (preferential exclusion), a protein is stabilized. This preferential exclusion mechanism could lead to protein−protein interactions effectively becoming more attractive and accelerating the aggregation kinetics.[20]

Arg^+ is a zwitterion and has unique physicochemical properties, i.e., electrostatic and hydration, compared to the simple salt ion and other amino acids: (1) Arg^+ is always used in a salt form with an anion to maintain the appropriate pH value. Arg^+ is close to a monovalent cation in the pH range between 4.5 and 7.0 typically used in drug product formulation in the biopharmaceutical industry, where the C-terminal carboxylate, N-terminal amino, and side chain are typically charged because of their pK_a value of 1.8, 9.0, and 12.5,[21] respectively; (2) the positive-charged side chain is guanidinium-ion-like (Gdm^+-like) and weakly hydrated.[10] The physicochemical properties of the side chain from Arg^+ in conjunction with those of the anion may hold the key to explain its mechanisms of interactions with proteins.

Recently experimental and molecular dynamics simulation have shown that Arg^+ can interact with protein surface, heteroionically with anions, and homoionically with itself in solution: (1) at low concentrations, the Gdm^+-like side chain of Arg^+ could interact with the protein surface through a cooperative manner with the Gdm^+-like side chains and carboxylates on the protein surface;[22] (2) the side chain can interact with aromatic and charged residues due to cation−π interaction and salt-bridge formation;[23] (3) the side chain from Arg^+ could pair with similar-size anions of hydrogen-bond acceptors,[20,24] e.g. SO_4^{2-}. However, for ArgCl, it has been shown that there is little Arg^+/Cl^- interaction in solution and that they are free to interact with protein surface;[20] (4) Arg^+ can interact with itself through stacking on top of each other or hydrogen bonding between Gdm^+-like side chain and C-terminal carboxylate.[23] Therefore, the overall effect of Arg^+ on protein thermal stability and aggregation kinetics can depend on the balance of homoion interactions, Arg^+−anion (heteroionic) interaction, and Arg^+−protein interactions, in addition to anion−protein interactions. Currently, the thermodynamic and kinetic ramifications of all these intertwined interactions in Arg^+ salt solution still remains to be studied systematically for aggregation and thermal stability of proteins with different biophysical properties, e.g., size and hydrophobicity, despite the recent progress made on smaller size proteins.[6,20,23,24]

In this report, we systematically studied the aggregation kinetics and thermal stability behavior for an IgG1 molecule at pH 4.8 below its pI of 8.8, where the antibody carries net positive charges, in four arginine salt solutions including Glu^-, acetate, Cl^-. and SO_4^{2-} as the anion. We evaluated the thermal unfolding of this antibody by both DSC and DSF, and then assessed its aggregation kinetics indirectly by calculating the reversibility of the C_H2 domain during the thermal unfolding in the DSC repetitive scan and then directly by an isothermal aggregation study monitored by size exclusion chromatography. There is an interest to study this pH condition of 4.8 because the majority of the antibodies in the biopharmaceutical industry have basic pI values and most of antibodies are formulated at pH conditions below the pI.

Our motivation is to explore how hydration and electrostatic properties of an anion could tune the effect of Arg^+ on the protein behavior. Acetate ion is monovalent and strongly hydrated, while the divalent SO_4^{2-} is with high charge density and strongly hydrated, with Cl^- being monovalent and slightly weakly hydrated.[10] Glu^- is close to a monovalent anion at pH 4.8 and considered as excluded from the protein surface.[25] From our experiments, the effect of Arg^+ can be readily compared to that of Na^+ in the solutions of the Cl^- and SO_4^{2-} salts as the benchmark. This fundamental study can help understand the effect of Arg^+ salts on protein behavior in general and could serve as a guide for biopharmaceutical formulation design where either attractive protein−protein interaction is preferred for crystalline formulation or repulsive protein−protein interaction is desired in liquid formulation for slow aggregation kinetics.

MATERIALS AND METHODS

Preparation of the Antibody Stock Solution. The IgG1 antibody with an experimentally measured pI of 8.8 made by MedImmune (Gaithersurg, MD) at 100 mg/mL was dialyzed into Mill-Q water using Slide-A-Lyzer with MWCO of 10,000 Da (ThermoFisher Scientific, Grand Island, NY). The volume

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ratio of the protein solution to water was ~1:100 with three exchanges over a period of 48 h at 4 °C. The material was then collected, and an antibody stock solution at 60 mg/mL was obtained.

Preparation of the Antibody Solution in Different Salt Solutions. All the chemicals were purchased with the highest purity grade possible. A base solution of 100 mM sodium acetate at pH 4.8 was prepared by mixing 50 mmol of NaOH with 100 mmol acetic acid in a final volume of 1 L. To prepare the individual stock solution of 1.0 M NaCl and 0.5 M Na_2SO_4 in 100 mM sodium acetate at pH 4.8, 1 mol of NaCl, and 0.5 mol of Na_2SO_4 were weighed and dissolved in water, respectively, followed by mixing the same amount of NaOH and acetic acid as used in the base solution and adjusting the volume to 1 L. Then the individual 1.0 M ArgCl, ArgGlu, and ArgAcetate in 100 mM sodium acetate pH 4.8 was prepared by mixing 1 mol of arginine base with 1 mol of hydrochloric acid, glutamic acid, or acetic acid, respectively, and adding the same amount of NaOH and acetic acid used in the base solution, followed by adjusting the volume to 1 L. Then 0.5 M Arg_2SO_4 in 100 mM sodium acetate pH 4.8 was prepared similarly but with the addition of 0.5 mol of sulfuric acid. Antibody samples (1 mg/mL) in different salt concentrations were prepared by adding 17 μL of the antibody stock solution to the 1.0 mL solution of the desired salt concentration, prepared from mixing the base solution and the salt stock solution.

Thermal Unfolding Experiments by DSC and DSF. Thermal unfolding transitions for the 1 mg/mL antibody samples were conducted in a nano-DSC (differential scanning calorimeter) (TA Instrument, Inc., New Castle, DE) using a scan rate of 1 °C/min from 10 to 90 °C in the full scan. Thermal reversibility of the antibody was studied by repeating the individual scan to 60 °C up to eight times in the separate experiments. Between the scans, the sample was cooled to 10 °C. The DSF experiment was conducted on a thermal cycler (Bio-RAD, Hercules, CA), and the Sypro Orange stock solution was mixed well with the antibody sample in a final volume ratio of 1:1000. Fluorescence was measured continuously at 1 Hz, while temperature was gradually raised at 0.2 °C/min. The derivative of relative fluorescence unit against temperature was then plotted so that the temperature where the derivative reached the first maximum is perceived as the melting temperature (T_m) of the C_H2 domain.

A previously developed mathematical model[18] was applied to fit the DSC data, i.e., molar heat capacity C_p vs temperature curves. The reversibility was calculated by the ratio of fitted C_p value in the third scan to that of the first scan. The mathematical model and fitting parameters used are essentially the same as described in the section of Description of the Model for Theoretical Thermodynamic and Kinetic Treatment under Materials and Methods from previous work.[18] The model fitting was performed with the programs written in MATLAB from MathWorks. Examples of the model fitting for the repetitive scans to calculate the reversibility are shown in Supporting Information.

Isothermal Aggregation Study by SEC-HPLC. The kinetics of the aggregation was conducted by dividing a protein solution at 1.0 mg/mL into several polypropylene Eppendorf tubes (containing 110 μL of sample) and placing the samples in a preheated water bath at 60 °C. At different time intervals, one tube was pulled from the water bath and immediately submerged in ice for at least 15 min. The sample was then quickly vortexed and centrifuged, and the monomer fraction measured by using size-exclusion chromatography. The method analyzed 60 μL of the sample on a 1290 HPLC system (Agilent, Santa Clara, CA) monitoring at 280 nm with an isocratic flow rate at 1 mL/min with the mobile phase of 100 mM sodium phosphate and 100 mM sodium sulfate at pH 6.8) over a TOSOH BIOSEP TSK-GEL G3000SWXL column (5 μm particle size, 7.8 × 300 mm) (Tosoh Bioscience, King of Prussia, PA) coupled to a guard column (TOSOH BioSEP TSK SW XL Guard Column). The monomer fraction for all the samples was calculated by dividing the monomer area of the sample by the total area of the unheated sample. Then a linear fit through all the data points at a designated salt concentration was performed to estimate the rate constant of the aggregation to compare the salt effect.

RESULTS

Thermal Stability of the C_H2 Domain by Both DSC and DSF. The thermal unfolding temperature by DSC, T_m, is normally the temperature at the center of enthalpic transition where 50% of the protein remains native and the rest is thermally unfolded. T_m is generally considered as a measurement of thermal stability of a protein. However, DSF measures the gradual rise in fluorescence intensity as the Sypro Orange molecule progressively binds to exposed nonpolar core of a protein when thermal unfolding continues. Figure 1a,b are the DSC thermograms and DSF data for this antibody at pH 4.8, respectively, in the selected salt conditions. In the buffer alone as shown in Figure 1a, there are three distinct thermal

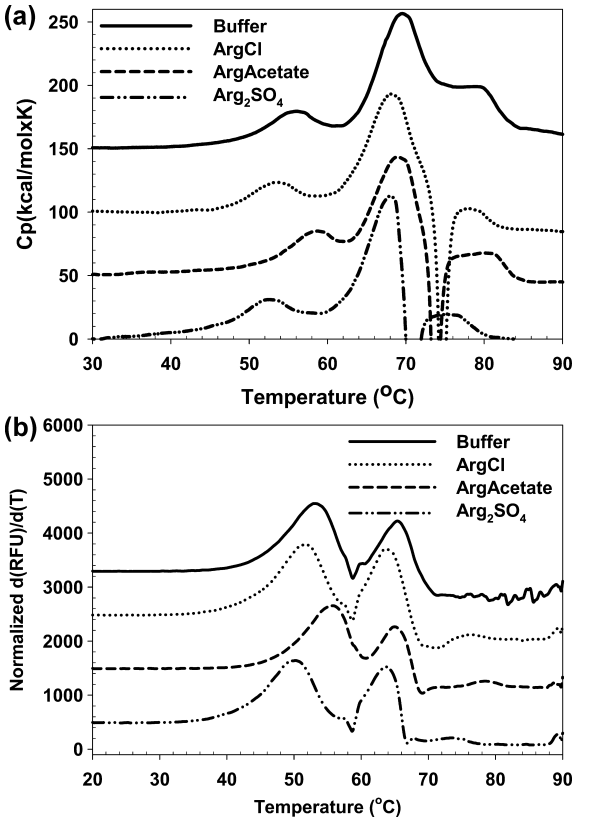

Figure 1. Thermal unfolding of the antibody at 1.0 mg/mL at pH 4.8 in the buffer, 100 mM ArgCl, 100 mM ArgAcetate, and 50 mM Arg_2SO_4 by DSC (a) and DSF (b). The apex of the first transition from each measurement is used as the unfolding temperature of the C_H2 domain for comparison purpose.

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unfolding transitions with T_m at ~56, 70, and 80 °C, respectively. Typically the transition with the lowest T_m is associated with the C_H2 domain of a monoclonal antibody. In Figure 1b there are two broad thermal unfolding transitions with the T_m, defined as the temperature where the d(RFU)/dT reaches maxima, at ~54 and 66 °C. The difference in the melting temperature by DSC and DSF reflects the selectivity difference between two techniques as aforementioned. The closeness of the T_m for the first thermal unfolding transition in the DSF run to the T_m for the first transition in the DSC scan suggests that the first transition in the DSF data could be from the C_H2 domain.

Both the DSC and DSF data in Figure 1 clearly demonstrate the anion specific effects in the Arg^+ salts on the stability of the C_H2 domain. As shown in Figure 1a, the addition of 100 mM ArgCl and 50 mM Arg_2SO_4 reduces the C_H2 T_m to ~54 and ~53 °C, respectively, while the addition of 100 mM ArgAcetate increases the C_H2 T_m to ~58 °C, in comparison to the buffer alone. DSF measurements as shown in Figure 1b show the same trend.

The anion-specific effects on the stability of the C_H2 domain at different salt concentrations are apparent as shown in Figure 2 by both (a) DSC and (b) DSF results. The most striking feature is the consistent improvement in thermal stability by both ArgGlu and ArgAcetate in contrast to the deterioration by ArgCl and Arg_2SO_4, highlighting the dominant contribution of the anion in the Arg^+ salts. However, it is surprising to see the perturbation on the thermal stability by ArgCl and NaCl in both DSC and DSF data is somewhat equivalent initially and up to the cation concentration of 75 mM, and begin to diverge at 100 mM, suggesting that there could be multiple mechanisms of Arg^+ and protein interactions. This trend is absent for Arg_2SO_4 and Na_2SO_4. These observations once again emphasize the dominant anion-specific effect by both Cl^- and SO_4^{2-} on the thermal stability at low concentrations. Contribution by the cation, i.e., Arg^+ versus Na^+, begins to surface at high salt concentrations for the Cl^- salts. The destabilization of the C_H2 domain by both Na_2SO_4 and Arg_2SO_4 is an important discovery because SO_4^{2-} is typically considered as a kosmotrope and stabilizes proteins in general.[10]

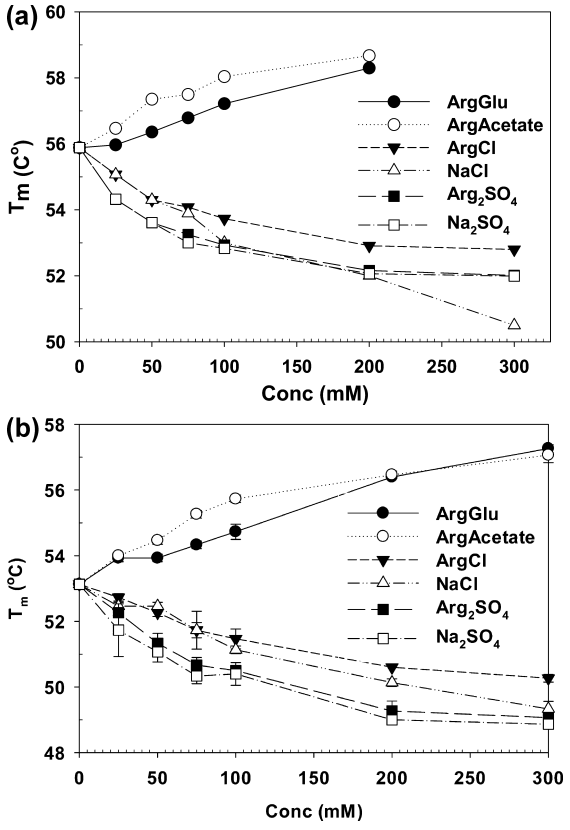

Figure 2. T_m results for the 1.0 mg/mL antibody at pH 4.8 in different salt concentrations of ArgGlu, ArgAcetate, ArgCl, NaCl, Arg_2SO_4, and Na_2SO_4 as measured by (a) DSC and (b) DSF (n = 3 replicates), respectively. The cation concentration of the studied salts is plotted on the X-axis.

In summary, the current study identifies the destabilizing effect of salt on the thermal stability of the mAb to follow the order of $Arg_2SO_4 \cong Na_2SO_4$ > ArgCl ≅ NaCl > ArgGlu > ArgAcetate, while the ranking for NaCl and Arg_2SO_4 begin to switch at 100 mM.

Reversibility in DSC Repetitive Scans. Reversibility is an indirect quantitative measurement of how quickly the antibody forms the irreversible aggregates during the DSC scan after the C_H2 domain is thermally unfolded.[18] Specifically, faster aggregation of the thermally unfolded state should result in worse reversibility in the repetitive scans. Qualitatively as shown in Figure 3a at pH 4.8 in 12.5 mM Arg_2SO_4, the amount of enthalpy to unfold the C_H2 domain up to 60 °C continuously decreases from the first scan to the third and fifth scans, which suggest the progressive loss of the native antibody due to aggregation during the repetitive scans. In comparison, in 25 mM ArgGlu solution as shown in Figure 3b, it appears that the extent of the drop is much shallower from scan 1 to scans 3 and 5. The reversibility for all salts studied is shown in Figure 3c. Apparently, there is both cation- and anion-specific effects on the reversibility behavior, e.g., ArgCl versus NaCl and ArgAcetate versus ArgCl. The addition of ArgGlu and ArgAcetate maintains the reversibility of the C_H2 domain fairly well, while the addition of the other salts decreases the reversibility in the order of Na_2SO_4 > Arg_2SO_4 > NaCl > ArgCl up to 50 mM. Furthermore, the reversibilities in the Arg^+ salts are consistently higher than the corresponding Na^+ salts. In addition, the reversibility in the Arg_2SO_4 becomes somewhat better than in the NaCl solution at 100 mM cation concentration. For example, there was still approximately 62% reversibility at 50 mM Arg_2SO_4, but the baseline in the rescan of the thermogram for the 100 mM NaCl condition was very poor due to fast aggregation, and therefore, no calculation of the reversibility can be made.

Isothermal Aggregation Study. In order to confirm the hypothesis that the worsening of reversibility in the DSC scan is due to aggregate formation, the effect of Arg^+ salts on the antibody aggregation kinetics up to 30 min were studied by incubating the antibody solutions at 60.0 °C above the T_m of the C_H2 domain at the respective salt concentrations, followed by the SEC-HPLC analysis. The dominant role of anion specific effect is readily discernible in Figure 4a. Both ArgGlu and ArgAcetate at 75 mM outperform the others with the slowest aggregation kinetics. Furthermore, the Arg^+ salts are consistently more effective at slowing down the aggregation kinetics than their Na^+ salt counterparts. The rate constants at all the salt concentrations studied for aggregation kinetics are plotted in Figure 4b for better illustration. At salt concentrations below 75 mM, the salts follow the order of Na_2SO_4 > Arg_2SO_4 > NaCl > ArgCl > ArgAcetate > ArgGlu for accelerating aggregation kinetics, in general agreement with the trends from the

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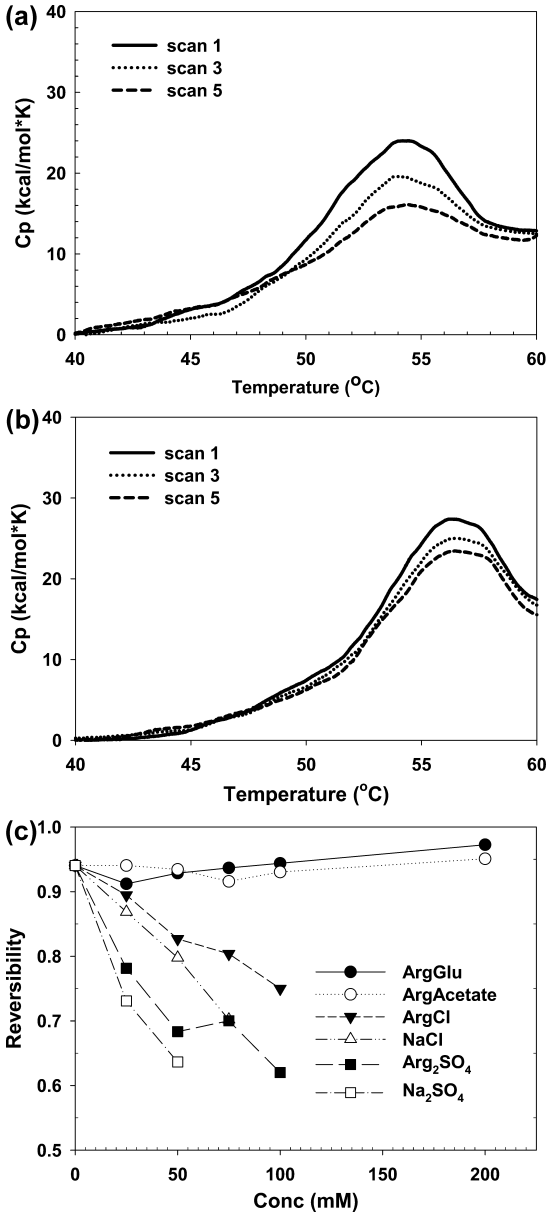

Figure 3. Reversibility studies for the 1.0 mg/mL antibody at pH 4.8 in ArgGlu, ArgAcetate, ArgCl, NaCl, Arg_2SO_4, and Na_2SO_4 at different concentrations. (a) Representative repetitive scans for the 1.0 mg/mL antibody at pH 4.8 in 12.5 mM Arg_2SO_4. (b) Representative repetitive scans for the 1.0 mg/mL antibody at pH 4.8 in 25 mM ArgGlu; (c) reversibility comparison. The cation concentration of the studied salts is plotted on the *X*-axis. Reversibility data is not shown for some of the salts at high concentration, e.g., 100 mM NaCl, due to noisy baseline in the repetitive scans, and the calculation cannot be performed.

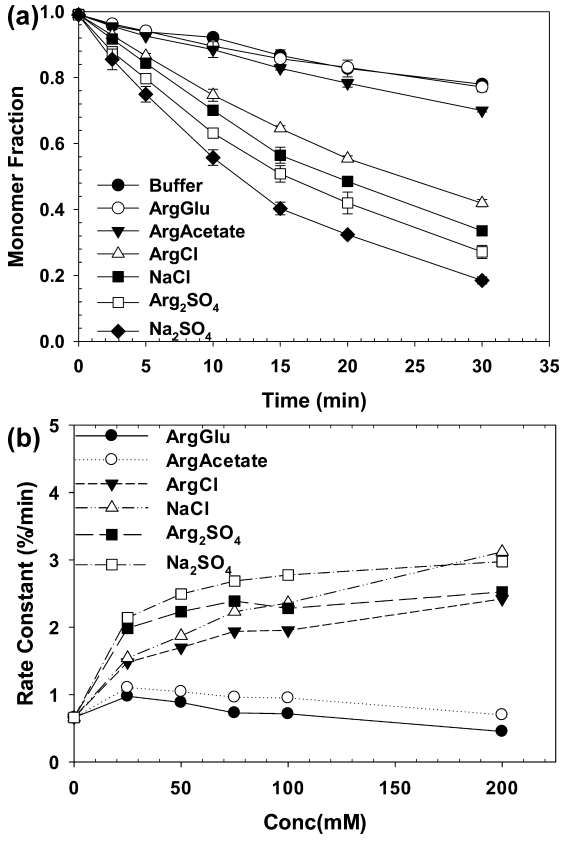

Figure 4. Isothermal aggregation kinetics study at 60 °C of 1 mg/mL antibody at pH 4.8 by size exclusion chromatograph in ArgGlu, ArgAcetate, ArgCl, NaCl, Arg_2SO_4, and Na_2SO_4, respectively. (a) Average monomer fractions (n = 3 independent runs) in the salt concentration of 75 mM for the monovalent salts and 37.5 mM for the divalent salts. (b) Calculated rate constant for the individual salt. The cation concentration of the studied salts is plotted on the *X*-axis.

reversibility study described above. Furthermore, as shown in Figure 4b, NaCl begins to switch its position with Arg_2SO_4 at 100 mM concentration level. At 200 mM, the salts now follow the order of $Na_2SO_4 \cong$ NaCl > ArgCl $\cong$ Arg_2SO_4 > ArgAcetate > ArgGlu for accelerating the aggregation kinetics. This switching event is consistent to what occurred in the DSC reversibility experiment. This finding points out the fact that the ranking of the salts on protein aggregation kinetics is not only anion dependent but also salt concentration dependent.

DISCUSSION

Inter- and Intramolecular Electrostatic Interactions Revealed by Na_2SO_4. The faster aggregation kinetics and more destabilization of the C_H2 domain in Na_2SO_4 compared to NaCl for this antibody suggest the contribution of intermolecular repulsive electrostatic interactions in aggregation behavior and intramolecular electrostatic interactions in maintaining protein stability, consistent with the previous findings for F_c in the same salt solutions.[18] At pH 4.8 below the pI of 8.8, the antibody molecule carries net positive charges. Intermolecular interactions could be dominated by repulsive electrostatic interactions between the protein molecules. Therefore, the aggregation kinetics is slow in the buffer alone as shown in both reversibility DSC repetitive scans in Figure 3c and isothermal aggregation kinetics study in Figure 4a. The acceleration of aggregation kinetics upon the addition of both Na_2SO_4 and NaCl as shown in Figure 4b suggests that the interactions of the ions, i.e., SO_4^{2-} and Cl^-, with the positive charged antibody are stronger than that of Na^+, thereby neutralizing the positive charges on the antibody and attenuating the electrostatic repulsive intermolecular interactions. The faster aggregation kinetics in Na_2SO_4 than in NaCl is consistent with the fact that the divalent SO_4^{2-} has higher charge density than the monovalent Cl^-, and therefore, it is more effective at neutralizing the positive charges on the antibody surface and attenuating the electrostatic repulsion.

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Our findings are in agreement with vibrational sum frequency spectroscopy measurement of the binding of SO_4^{2-} and Cl^- to the positive-charged surface of BSA at pH below its pI: specifically, the binding of SO_4^{2-} leads to more attenuation of interfacial water structure than Cl^- due to charge neutralization.[15]

The denaturing behavior of Na_2SO_4 in this study and the F_c experiment[18] strongly suggests that intramolecular electrostatic interactions may play an essential role in maintaining the stability of the C_H2 domain. The reason is that unlike the promiscuity of chaotropic anions of Cl^- and SCN^-, SO_4^{2-} is a strongly hydrated anion with high charge density and therefore extremely selective for positive-charged side chains, but not peptide bonds in the backbone of proteins.[11] Therefore, it may destabilize the C_H2 domain peculiarly through either disruption of intramolecular ion pairing (or salt bridges) or binding of exposed positive-charged residues upon unfolding. Furthermore, the more destabilization of the C_H2 domain by Na_2SO_4 than NaCl at low salt concentration further supports that SO_4^{2-} interacts electrostatically with the antibody more strongly than Cl^-. The interaction between SO_4^{2-} and the antibody in both native and unfolded states on a residue level will be the subject of our future investigation.

Therefore, the prominent features described above could exemplify the antibody aggregation behavior in general: (1) the binding of an anion, i.e., SO_4^{2-} and Cl^-, to a positive-charged protein at pH below its pI could lead to fast aggregation kinetics; (2) the unfolding of the C_H2 domain alone, the least thermally stable domain of an antibody molecule, can induce antibody aggregation.

Arg^+ Binds More Strongly to the Antibody than Na^+. The most striking feature in Figure 4b is the domination of anion on the aggregation kinetics of the positive-charged antibody in Arg^+ salt solutions, as illustrated by the observation that the difference in the rate constant is much more drastic between ArgCl and ArgAcetate than between NaCl and ArgCl. The anion-dominant feature is consistent with the Law of Matching Water Affinities:[10] (1) the strongly hydrated anions, e.g., acetate and Glu, do not interact significantly with the weakly hydrated/positive charged protein surface in comparison to the weakly hydrated anions, e.g., Cl^-; (2) Arg^+ should be more free to interact with the protein surface in solution, when its counterion from its salt form is strongly hydrated since Arg^+ itself is weakly hydrated. In addition, it is reasonable to hypothesize that both SO_4^{2-} and Cl^- interact with the antibody surface more strongly than Arg^+ because the addition of Arg_2SO_4 and ArgCl attenuates the repulsive protein−protein interaction as suggested by the acceleration of aggregation kinetics. Furthermore, the ability of Arg^+ to slow down the aggregation kinetics versus Na^+ consistently in both Cl^- and SO_4^{2-} salts strongly suggests that the binding for Arg^+ to the antibody surface could be stronger than that of Na^+. This is consistent with the theory that Na^+ typically has weak interaction with protein surface and is especially expelled from a positive charged protein surface,[15] while Arg^+ can possibly interact with protein surface through the cooperative binding with the Gdm^+-like ion and carboxylate groups on protein surface and cation−π interaction with aromatic side chains.[23] This mechanism could conceptually lead to the suppression of aggregation kinetics: (1) the colloidal stability of the unfolded antibody could improve, i.e., aggregation suppression, suggested by Wyman's theory of thermodynamic linkage: this binding results in a deeper drop in the free energy for a free molecule in solution versus the associated molecules like in the precipitated form; or (2) the positive-charged nature of Arg^+ could effectively strengthen the electrostatic repulsion between the positive-charged antibody molecules[13] and slow the aggregation kinetics in comparison to Na^+.

ArgCl and Arg_2SO_4 as Denaturants, Dominated by Anions, Possibly through Multiple Mechanisms. It is interesting to note that the stronger interaction of Arg^+ with the positive charged protein surface than that of Na^+ does not translate into the more destabilization of the C_H2 domain for this antibody at low salt concentrations up to 75 mM, in Figure 2a, i.e., ArgCl and NaCl as a pair, Arg_2SO_4 and Na_2SO_4 as the other pair. The behavior of the antibody in ArgCl solution is in agreement with other findings that ArgCl is more of an aggregation suppressor, rather than a stabilizer.[20] Thermodynamically, the similarity in the effect on thermal stability by the pairs of ArgCl and NaCl, Arg_2SO_4 and Na_2SO_4 would suggest that the further drop of the free energy for both the N and U states due to stronger interactions of Arg^+ is equally deep, and therefore, the free energy difference between N and U in either Arg_2SO_4 or ArgCl solution is similar to that in respective Na^+ salt solution at the same cation concentration. Mechanistically, this observation implies that Arg^+ below 75 mM may mostly interact with the antibody surface without preference for the exposed backbone upon thermal unfolding. Otherwise, the interaction with the exposed backbone could lead to greater drop in thermal stability. The destabilization by Arg_2SO_4 again confirms that (1) the contribution of electrostatic interaction in maintaining the thermal stability of the C_H2 domain; (2) the interaction of SO_4^{2-} with the antibody surface outweighs that of Arg^+. It remains a future research topic of how Arg^+ could not perturb this intramolecular electrostatic stabilization more strongly than Na^+, despite the fact that Arg^+ interacts with the protein surface more strongly than Na^+.

It is noted that the effect by ArgCl and NaCl on T_m began to diverge at 75 mM as shown in Figure 2a. It has been shown that Arg^+ and Cl^- are mostly free from heteroion interactions, but there is homoion interaction between Arg^+ molecules through either stacking on top of each other or hydrogen bonding through the Gdm^+-like side chain and carboxylate.[20,23] As the concentration of ArgCl increases, Arg^+ could begin to form large size clusters through heteroion interaction and be preferentially excluded to the bulk solution, rather than the binding to the protein surface at the low salt concentration.[20,23] Therefore, this preferential exclusion could begin to stabilize the C_H2 domain and counter the continuous destabilization by Cl^-.

The destabilizing behavior by Arg_2SO_4 is similar to that of Na_2SO_4 throughout the concentrations studied. It should be noted that at 200 mM Na^+ NaCl still destabilizes the C_H2 domain, while the effect by Na_2SO_4 begins to level off. This divergent behavior occurred in the F_c study as well.[18] This could be due to the protein stabilization effects from either the preferential exclusion of the strongly hydrated SO_4^{2-} from the protein surface or competition for interfacial water molecules on the protein surface by SO_4^{2-} against the protein surface.[18] However, Cl^- could not only disrupt the mechanisms of electrostatic stabilization but also bind the exposed peptide backbone, resulting in continuous destabilization of the C_H2 domain. Unlike for ArgCl, there are strong heteroion interactions between Arg^+ and SO_4^{2-} through hydrogen bonding in Arg_2SO_4 solutions. Consequently, the heteroion pairing at high concentration of Arg_2SO_4 could limit the ability

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of SO_4^{2-} to compete for interfacial water molecules from the protein surface. Therefore, there is less extent of stabilization by SO_4^{2-} through preferential exclusion in Arg_2SO_4 than in Na_2SO_4; but the preferential exclusion of the clustering of Arg^+ could compensate for the loss of stabilization by SO_4^{2-}. These two competing mechanisms might explain why Arg_2SO_4 is similar to Na_2SO_4 in destabilizing the C_H2 domain of the antibody.

Stabilization by ArgGlu and ArgAcetate Are Accompanied by pH Increase. This stabilization by ArgGlu in our study is consistent with the literature for monoclonal antibodies;[26] however, the stabilization by ArgAcetate is opposite to what was reported for bovine α-chymotrypsinogen A Type II (aCgn).[20] We discovered that the addition of these salts increased the pH significantly, e.g., to ~5.4 from 4.8 with the addition of 200 mM ArgAcetate (see Figures (III) and (IV) in Supporting Information). The stabilization of the C_H2 domain accompanied by this apparent pH increase is consistent with the other findings that thermal stability of the F_c generally improves as pH moves toward its pI.[18] However, this pH effect could be confounded by other mechanisms between the ion and protein surface. Specifically, the C_H2 domain can be stabilized by the following multiple mechanisms of ion−protein interactions: (1) the strongly hydrated anions, i.e., acetate and Glu^-, are most likely repelled from the antibody surface and cannot disrupt the electrostatic interactions for stabilizing the C_H2 domain; (2) preferential exclusion could, in turn, stabilize the antibody.

Protein Identity Matters in Evaluating the Effects by Arg Salts. The destabilization pattern for the C_H2 domain in ArgCl shown in Figure 2a is consistent with what was reported for lysozyme,[22] ovalbumin,[22] and aCgn,[20] but the antibody destabilization by Arg_2SO_4 in our study is contrary to what has been reported for aCgn (pI: ~8.8) at pH 5.[20] Also the acceleration of protein aggregation in both Arg_2SO_4 and ArgCl versus the buffer alone for this antibody is inconsistent with what has been reported for aCgn,[20] where the addition of both salts slowed down the protein aggregation kinetics. These complex data highlight the importance of the individual protein structure, especially the mechanism of maintaining the protein folding or stability, in dictating the effects by the Arg^+ salts. The contrasting effect by a cosolute was illustrated by studying the unfolding behavior of alanine-based helical peptides stabilized through hydrogen bonds versus tryptophan zipper (trpzip) peptides stabilized largely through cross-strand indole−indole interactions in both GdmCl and Gdm_2SO_4 solutions. While both the trpzip peptides and alanine-based α-helical peptides were sensitive to GdmCl denaturation, the trpzip peptides were unaffected by Gdm_2SO_4 and the alanine-based α-helical peptides can be denatured by Gdm_2SO_4.[27] The most important discovery in our work is the destabilization of the C_H2 consistently by both Arg_2SO_4 and Na_2SO_4, which strongly suggests that the C_H2 domain could be stabilized electrostatically. In the case of aCgn, it was hypothesized that sulfate was excluded from the protein surface, and therefore, a stabilization effect was observed.

CONCLUSIONS

Our study clearly demonstrates the dominating role of the anion in the Arg salts in these two events: (1) the C_H2 thermal stability; (2) aggregation kinetics. However, the effect of Arg^+ in suppressing aggregation kinetics is discernible from that of Na^+ for a net positive-charged monoclonal antibody molecule. Also, the addition of all the salts in general accelerates the aggregation kinetics. The destabilization of the C_H2 domain by both Arg_2SO_4 and Na_2SO_4 strongly suggests that electrostatic interactions contribute to the stability of the C_H2 domain, and, therefore both strongly hydrated monovalent anions, i.e., acetate and Glu^-, cannot effectively disrupt because of their exclusions from the protein surface. The presence of stronger electrostatic interactions between the antibody and SO_4^{2-} is supported by the fastest aggregation kinetics in the pair of NaCl and Na_2SO_4 and the pair of ArgCl and Arg_2SO_4, where the binding of the anions neutralizes the positive charges of the protein and attenuates the repulsive electrostatic interactions between the antibody molecules. The consistent pattern of Arg^+ to slow down the aggregation kinetics more than Na^+ suggests that Arg^+ binds to the protein surface stronger than Na^+, but this mechanism does not result in significant destabilization of the C_H2 domain, suggesting that Arg^+ interacts mostly with the protein surface, rather than the exposed backbone upon unfolding. Finally, our work highlights the important roles of the interactions between Arg^+ and a positive-charged protein, the interactions between anions and the protein, homoionic Arg^+−Arg^+ interactions, and heteroionic Arg−anion interactions on antibody thermal stability and aggregation kinetics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00255.

DSC thermograms and pH measurements (PDF)

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