Quantitative Analysis of the Effect of Salt Concentration on Enzymatic Catalysis

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Abstract: Like pH, salt concentration can have a dramatic effect on enzymatic catalysis. Here, a general equation is derived for the quantitative analysis of salt−rate profiles: $k_{cat}/K_M = (k_{cat}/K_M)_{MAX}/[1 + ([Na^+]/K_{Na^+})^{n'}]$, where $(k_{cat}/K_M)_{MAX}$ is the physical limit of k_{cat}/K_M, K_{Na^+} is the salt concentration at which $k_{cat}/K_M = (k_{cat}/K_M)_{MAX}/2$, and $-n'$ is the slope of the linear region in a plot of $\log(k_{cat}/K_M)$ versus $\log [Na^+]$. The value of n' is of special utility, as it reflects the contribution of Coulombic interactions to the uniform binding of the bound states. This equation was used to analyze salt effects on catalysis by ribonuclease A (RNase A), which is a cationic enzyme that catalyzes the cleavage of an anionic substrate, RNA, with k_{cat}/K_M values that can exceed 10^9 M^{-1} s^{-1}. Lys7, Arg10, and Lys66 comprise enzymic subsites that are remote from the active site. Replacing Lys7, Arg10, and Lys66 with alanine decreases the charge on the enzyme as well as the value of n'. Likewise, decreasing the number of phosphoryl groups in the substrate decreases the value of n'. Replacing Lys41, a key active-site residue, with arginine creates a catalyst that is limited by the chemical conversion of substrate to product. This change increases the value of n', as expected for a catalyst that is more sensitive to changes in the binding of the chemical transition state. Hence, the quantitative analysis of salt−rate profiles can provide valuable insight into the role of Coulombic interactions in enzymatic catalysis.

Introduction

Coulombic interactions[1] contribute to the stability of many receptor−ligand complexes in aqueous solution.[2−8] The formation of an ion pair liberates immobilized ions and water molecules, thus increasing the overall entropy. Ion-pair formation is less favorable in solutions that contain a high concentration of salt, and the effect of salt concentration on receptor−ligand interactions is well known.[1−8] Here, we develop the first quantitative model to describe the effect of salt concentration on the binding and turnover of a substrate by an enzyme. We test the validity of our model by analyzing the effect of salt concentration on catalysis by ribonuclease A (RNase A;[9] EC 3.1.27.5).

RNase A has been archetypal for the characterization of Coulombic interactions between a protein and a single-stranded nucleic acid.[10−12] RNase A is a pyrimidine-specific ribonuclease from bovine pancreas.[13,14] A series of cationic residues on the surface of the enzyme can form extensive Coulombic interactions with the phosphoryl groups of a nucleic acid. The cationic residues comprising specific phosphoryl group-binding sites have been revealed by X-ray diffraction analyses.[15−18] Four subsites have been identified: P(−1), P0, P1, and P2, as shown in Figure 1A. Arg85 and Lys66 comprise the P(−1) and P0 sites, respectively.[12,19] The active-site residues His12, Lys41,

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(1) We use the term "Coulombic" rather than "electrostatic" to describe the interaction between two point charges. We consider "electrostatic" to be a general term that encompasses Coulombic interactions, hydrogen bonds, and dipole−dipole interactions, and "Coulombic" to be a specific term that refers only to interactions that obey Coulomb's law: $F = q_1q_2/(4\pi\epsilon r^2)$. (a) Coulomb, C. A. *Collection de Mémoires Relatifs a la Physique*; Gauthier-Villars: Paris, 1884. (b) Gillmor, C. S. *Coulomb and the Evolution of Physics and Engineering in Eighteenth-Century France*; Princeton University Press: Princeton, NJ, 1971.

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(9) Abbreviations: 6-FAM, 6-carboxyfluorescein; 6-TAMRA, 6-carboxytetramethylrhodamine; Bistris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; cCMP, cytidine 2′,3′-cyclic phosphate; FRET, fluorescence resonance energy transfer; K_a, acid dissociation constant; NMR, nuclear magnetic resonance; PDB, protein data bank; RNase A, bovine pancreatic ribonuclease A; T_m, temperature at the midpoint of thermal denaturation.

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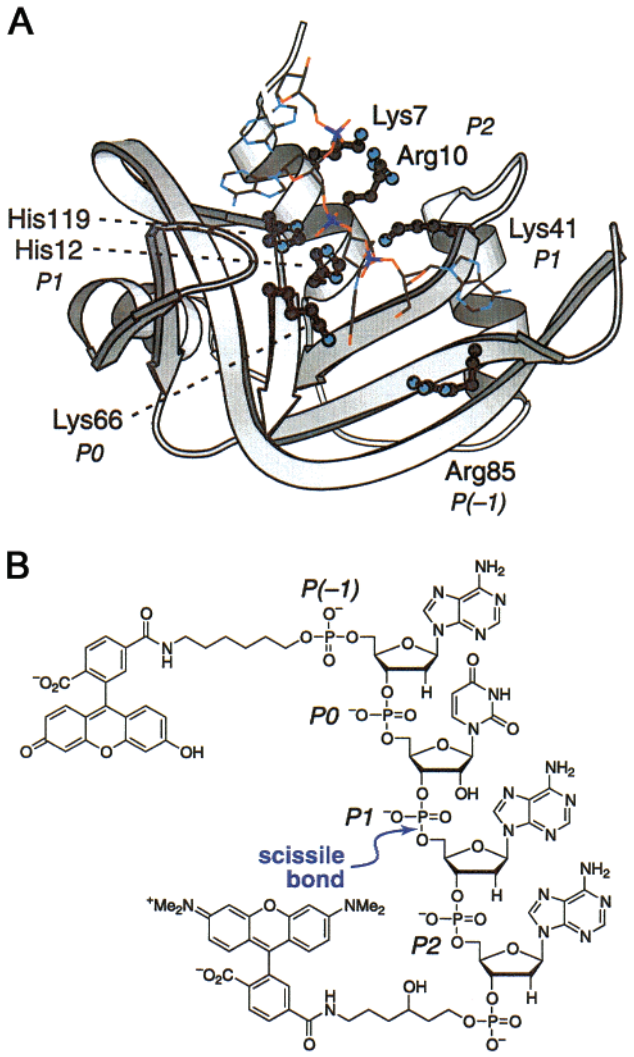

Figure 1. (A) Three-dimensional structure of the ribonuclease A−d(ApTpApApG) complex (PDB entry 1RCN[17]). The structure is depicted in a ribbon diagram made with the program MOLSCRIPT.[78] Cationic residues comprising phosphoryl group-binding subsites [P(−1), P(0), P(1), and P(2)] are shown in ball-and-stick representation with labels. The d(ApTpApApG) ligand is shown as a solid line. Electron density was not apparent for the guanosine 5′-phosphate residue of the ligand. (B) Structure of substrate **2** indicating the enzymic subsites that interact with its phosphoryl groups.[19,32,33]

and His119 comprise the P1 site. These residues have roles in binding to a phosphoryl group in the active site[20] as well as in catalysis.[21−24] Finally, Lys7 and Arg10 comprise the P2 site.[12,25]

The extensive Coulombic interactions in RNase A−nucleic acid complexes give rise to strong salt effects on the stability of these complexes. Twenty-five years ago, Jensen and von Hippel showed that the equilibrium association constant for RNase A and denatured DNA decreases from 5.6×10^5 to 1.2×10^4 M as $[Na^+]$ is increased from 0.030 to 0.070 M.[10] The logarithm of the observed equilibrium association constant shows a linear dependence on log $[Na^+]$. Concurrently, Record and co-workers successfully used polyelectrolyte theory to explain this thermodynamic salt effect.[11] Their analysis showed that the dependence of log K_{obs} on log $[Na^+]$ is linearly proportional to the number of ion pairs formed upon complex formation. The salt effects on RNase A complex formation implicated the involvement of about seven ion pairs in the interaction of the enzyme with denatured DNA. Previously, we used salt effects to characterize nucleic acid binding by RNase A variants with altered phosphoryl group-binding subsites.[12]

Salt effects on the kinetics, as well as the thermodynamics, of protein−nucleic acid interactions have been analyzed.[26,27] Moreover, the contribution of Coulombic interactions to stabilizing charged transition states in enzymatic catalysis has been appreciated.[2,28,29] Still, no quantitative model for salt effects on enzymatic catalysis exists. Here, we present such a model. We also acquire salt−rate profiles for catalysis by wild-type RNase A and its K7A/R10A/K66A and K41R variants. Lys7, Arg10, and Lys66 are proximal to phosphoryl groups of a bound substrate, and K7A/R10A/K66A RNase A displays greatly decreased Coulombic interactions with a nucleic acid.[12] Lys41 is critical for catalysis by RNase A, as it donates a hydrogen bond to the transition state.[21] Replacing Lys41 with an arginine residue does not decrease the number of Coulombic interactions with a substrate but impairs catalysis. We also analyze salt−rate profiles for three analogous substrates with a different number of phosphoryl groups. Our quantitative model of salt effects on enzymatic catalysis enables a meaningful interpretation of all of the salt−rate profiles.

Experimental Section

Materials. Wild-type RNase A and the H12A and K41R variants were produced, folded, and purified as described elsewhere.[21,30,31] K7A/R10A/K66A RNase A was a generous gift of B. M. Fisher. Substrates **1**−**3** were from Integrated DNA Technologies (Coralville, IA).

Substrate **1**	6-FAM∼rUdA∼6-TAMRA
Substrate **2**	6-FAM∼dArU$(dA)_2$∼6-TAMRA
Substrate **3**	6-FAM∼$(dA)_2$rU$(dA)_3$∼6-TAMRA

These substrates are DNA−RNA chimeras with a 5′ 6-carboxyfluorescein label (6-FAM) and 3′ 6-carboxytetramethylrhodamine (6-TAMRA) label, as depicted for substrate **2** in Figure 1B. When the substrate is intact, the fluorescence of the fluorescein moiety is minimal because of the quenching by fluorescence resonance energy transfer (FRET) to the rhodamine moiety. A large increase in fluorescence occurs upon cleavage of the P−$O^{5'}$ bond on the 3′ side of the single ribonucleotide residue embedded within the substrate.[32,33]

[Bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bistris) was from ICN Biomedicals (Aurora, OH). NaCl was from Fisher Scientific (Fair Lawn, NJ). Concentrations of wild-type RNase A and its variants

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were determined spectrophotometrically by using $\epsilon = 0.72$ mL mg^{-1} cm^{-1} at 277.5 nm.[34] Concentrations of the substrates **1**−**3** were determined spectrophotometrically by using $\epsilon =$ 76 340, 102 400, and 126 400 M^{-1} cm^{-1} at 260 nm, respectively.[32]

Assays of Enzymatic Activity. The ribonucleolytic activity of wild-type RNase A was determined at 23 °C with fluorogenic substrates **1**−**3** in 2.00 mL of 1.0 mM Bistris−HCl buffer containing NaCl (0.010−1.0 M), H12A RNase A (5.0 nM), and wild-type RNase A (0.050 nM). A 1.0 mM Bistris−HCl buffer was prepared by diluting 0.10 M Bistris−HCl (pH 6.0). The pH of the actual 1.0 mM Bistris−HCl buffers varies from pH 5.92 ([Na$^+$] = 0.010 M) to pH 6.12 ([Na$^+$] = 1.0 M) according to the salt concentration. Only a minimal concentration of buffer (1.0 mM) is used in this experiment. When a buffer component is cationic, Na$^+$ is not the only cation in the reaction, which introduces a complication in salt−rate profile analysis. Only when the concentration of buffer is much lower than [Na$^+$] can the contribution of the buffer cation be ignored and the salt−rate profile analyzed as a function of [Na$^+$]. The concentrations of substrates **1**−**3** in the reactions were 4.3, 20, and 19 nM, respectively. Substrate concentrations were low to ensure that the reaction follows the pseudo-first-order kinetics necessary for the determination of k_{cat}/K_M.

The determination of enzymatic activity in solutions of low salt concentration can be complicated by inhibition from contaminants in a buffer solution. We have reported that 0.025 M Bistris−HCl buffer does not show any significant inhibition to catalysis by RNase A.[35] Still, when the salt concentration was decreased to 0.010 M, inhibition was observed even with 1.0 mM Bistris−HCl buffer. Hence, H12A RNase A (5.0 nM) was added to the assay mixture to absorb any inhibitory contaminants without affecting catalysis.[35] Because H12A RNase A has >10^4-fold less ribonucleolytic activity than does wild-type RNase A,[22] its contribution to catalysis was negligible. For assays of the K41A and K7A/R10A/K66A variants, the enzyme concentration in the reactions was increased to 0.50 and 5.0 nM, respectively, because of the reduced catalytic activities of these variants. For assays of the K7A/R10A/K66A variant, H12A RNase A was not used in the assay mixture because the concentration of the K7A/R10A/K66A variant was already high enough to obviate inhibition by buffer contaminants.

Fluorescence was measured with a QuantaMaster 1 photon-counting fluorescence spectrometer from Photon Technology International (South Brunswick, NJ) using 493 and 515 nm as excitation and emission wavelengths, respectively. Assays were performed in quartz or glass cuvettes (1.0 cm pathlengh; 3.5 mL volume) from Starna Cells (Atascadero, CA). The value of V/K for reactions was determined by regression analysis of the fluorescence intensity change with eq 1 or 2, depending on whether the reaction reaches completion or not, as described previously.[32,33]

$$F = F_0 + (F_{max} - F_0)(1 - e^{-(V/K)t}) \quad (1)$$

$$V/K = \frac{(\Delta F/\Delta t)}{F_{max} - F_0} \quad (2)$$

In eqs 1 and 2, F_0 is the fluorescence intensity before a reaction is initiated, F_{max} is the maximum fluorescence intensity when the substrate in the reaction is fully cleaved, and $\Delta F/\Delta t$ is the slope of fluorescence intensity change for an initial linear region. The value of k_{cat}/K_M was calculated by dividing V/K by the enzyme concentration. The validity of the determined k_{cat}/K_M values was confirmed in two ways. First, the residual error from the curve-fitting to eq 1 was monitored carefully to ensure that the reaction followed pseudo-first-order kinetics. R^2 values for most fits were >0.99. Second, specifically at low salt concentration, k_{cat}/K_M was verified to be identical at two different concentrations of substrate to ensure that the substrate concentration was enough below K_M to allow for the direct determination of k_{cat}/K_M.

Results

Theory. A notional free energy profile of catalysis by RNase A is depicted in Figure 2. The rate constants k_1, k_2, and k_3 are for the association of the enzyme (E) and substrate (S), dissociation of the ES complex, and irreversible conversion of the ES complex to the EP complex, respectively. The second-order rate constant for the overall reaction, k_{cat}/K_M, is a function of these rate constants (see Supporting Information).

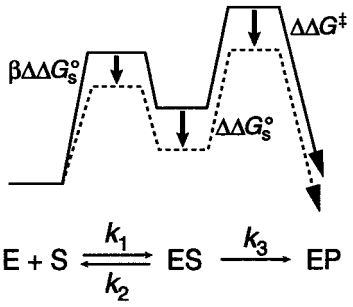

Figure 2. Effect of salt on a notional free energy profile of enzymatic catalysis. The solid line indicates the free energy profile when [Na$^+$] = 1.0 M; the dashed line indicates the free energy profile when [Na$^+$] < 1.0 M. To facilitate comparisons, each E + S state is set to the same free energy. k_1, k_2, and k_3 are rate constants for the association, dissociation, and chemistry steps, respectively; $\Delta\Delta G_S°$, $\Delta\Delta G^‡$, and $\beta\Delta\Delta G_S°$ indicate the effect of decreasing [Na$^+$] from 1.0 M on the free energy of the ES complex, the transition state for the chemistry step, and the transition state for the substrate-binding step, respectively.

$$k_{cat}/K_M = \frac{k_1 k_3}{k_2 + k_3} \quad (3)$$

The major effect of salt concentration on the free energy profile in Figure 2 is to change the free energy of the bound states (ES complex and transition state for the chemistry step) relative to that of the free states (unliganded E and S). According to studies of salt effects on the stability of RNase A−nucleic acid complexes,[11] the salt effect on the equilibrium association constant for the ES complex, $K_S = k_1/k_2$, can be written

$$\frac{\partial \log K_S}{\partial \log [Na^+]} = -n \quad (4)$$

where n is the number of counterions released from the substrate upon complex formation. For simplicity, it is assumed that Na$^+$ is the only cation in the system. By integrating eq 4, K_S can be written

$$K_S = K_S^{\ominus}[Na^+]^{-n} \quad (5)$$

where $K_S^{\ominus}$ is the equilibrium association constant of E and S when [Na$^+$] = 1 M (with the "⊖" superscript denoting the state in which [Na$^+$] = 1 M). The association free energy of the ES complex, $\Delta G_S°$, can be expressed as a function of [Na$^+$]:

$$\Delta G_S° = \Delta G_S^{\ominus} + nRT \ln [Na^+] \quad (6)$$

where $\Delta G_S^{\ominus}$ is the association free energy of the ES complex when [Na$^+$] = 1 M. Now, define $\Delta\Delta G_S°$ as the difference between $\Delta G_S°$ and $\Delta G_S^{\ominus}$:

$$\Delta\Delta G_S° = \Delta G_S° - \Delta G_S^{\ominus} = nRT \ln [Na^+] \quad (7)$$

$\Delta\Delta G_S°$ reports on the salt effect on the thermodynamic stability of the ES complex (Figure 2). By applying a linear free energy relationship, the change in the free energy of activation for the association step(s), $\Delta\Delta G_1^‡$, can be written

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$$\Delta\Delta G_1^{\ddagger} = \beta\Delta\Delta G_S^{\circ} \quad (8)$$

where β is a constant of proportionality between thermodynamic changes and kinetic changes to the association step(s) and has a value of $0 < \beta < 1$.[36−42] The change in the free energy of activation for the dissociation step, $\Delta\Delta G_2^{\ddagger}$, can then be written

$$\Delta\Delta G_2^{\ddagger} = \Delta\Delta G_1^{\ddagger} - \Delta\Delta G_S^{\circ} = (\beta - 1)\Delta\Delta G_S^{\circ} \quad (9)$$

By using eqs 7−9, k_1 and k_2 can be written as a function of $[Na^+]$:

$$k_1 = k_1^{\ominus}[Na^+]^{-\beta n} \quad (10)$$

$$k_2 = k_2^{\ominus}[Na^+]^{(1-\beta)n} \quad (11)$$

where $k_1^{\ominus}$ and $k_2^{\ominus}$ are the rate constants when $[Na^+] = 1$ M.

The rate constant for the chemistry step, k_3, can be approximated to be independent of the salt concentration of the medium. The major component of the salt effect on k_1 and k_2 is the effect of salt concentration on the entropy change of the medium caused by the release (or absorption) of counterions upon complex formation (or dissociation). The number of counterions associated with the substrate in the ES complex is unlikely to differ significantly from that associated with the transition state of the chemistry step. Indeed, k_{cat} for the hydrolysis of cytidine 2′,3′-cyclic phosphate (cCMP) by RNase A is insensitive to ionic strength.[43] Because k_{cat} for cCMP hydrolysis is determined mainly by the chemistry step,[44] it provides a sensitive probe for the effect of ionic strength on that step. Moreover, when the catalytic activity of RNase A is determined with a saturating concentration of cCMP (0.050 M), the enzyme retains more than half of its catalytic activity in an aqueous solution containing 50% (v/v) dioxane.[45] These data are consistent with the active site of the ES complex being excluded from the bulk solution, as is apparent in the structure of RNase A−nucleic acid complexes.[46−48]

By using eqs 10 and 11, k_{cat}/K_M can be expressed as a function of $[Na^+]$:

$$k_{cat}/K_M = k_1^{\ominus}[Na^+]^{-\beta n}\left(\frac{C_f}{1 + C_f}\right) \quad (12)$$

where

$$C_f = C_f^{\ominus}[Na^+]^{(\beta-1)n} \quad (13)$$

and $C_f = k_3/k_2$ is a forward commitment factor.[49,50] The dependence of k_{cat}/K_M on $[Na^+]$ can then be calculated from eqs 12 and 13:

$$\frac{\partial \log(k_{cat}/K_M)}{\partial \log [Na^+]} = -n\left(\frac{1 + \beta C_f}{1 + C_f}\right) \quad (14)$$

Because $\beta < (1 + \beta C_f)/(1 + C_f) < 1$,

$$-n < \frac{\partial \log(k_{cat}/K_M)}{\partial \log [Na^+]} < -\beta n \quad (15)$$

Thus, the slope in a $\log(k_{cat}/K_M)$ versus $\log [Na^+]$ plot varies from $-n$ to $-\beta n$, according to C_f.

Equation 12 is impractical to fit to a salt−rate profile, as the constants β and n are unlikely to be independent enough to be determined simultaneously in a regression analysis. This dilemma is resolvable if $\partial \log(k_{cat}/K_M)/\partial \log [Na^+]$ is approximated as a constant, $-n'$:[51]

$$\frac{\partial \log(k_{cat}/K_M)}{\partial \log [Na^+]} \approx -n' \quad (16)$$

where $\beta n < n' < n$. For a sluggish enzyme, $C_f \ll 1$, and n' would be close to n. For an enzyme limited by substrate association, $C_f \gg 1$, and n' would be close to βn. By integrating eq 16, k_{cat}/K_M can be written

$$k_{cat}/K_M = (k_{cat}/K_M)^{\ominus}[Na^+]^{-n'} \quad (17)$$

where $(k_{cat}/K_M)^{\ominus}$ is k_{cat}/K_M when $[Na^+] = 1$ M.

Although Coulombic forces are stronger at low $[Na^+]$, the value of k_{cat}/K_M is ultimately limited by the encounter of enzyme and substrate. The value of k_{cat}/K_M at this physical limit is $(k_{cat}/K_M)_{MAX}$. Thus, k_{cat}/K_M will be limited by the smaller of $(k_{cat}/K_M)_{MAX}$ and $(k_{cat}/K_M)^{\ominus}[Na^+]^{-n'}$,[52] as in

$$\frac{1}{k_{cat}/K_M} = \frac{1}{(k_{cat}/K_M)_{MAX}} + \frac{1}{(k_{cat}/K_M)^{\ominus}[Na^+]^{-n'}} \quad (18)$$

Equation 18 can be rewritten in a simpler form:

$$k_{cat}/K_M = \frac{(k_{cat}/K_M)_{MAX}}{1 + ([Na^+]/K_{Na^+})^{n'}} \quad (19)$$

where $K_{Na^+} = [(k_{cat}/K_M)^{\ominus}/(k_{cat}/K_M)_{MAX}]^{1/n'}$. Equation 19, rather than eq 17, is applicable to reactions that are limited by the encounter of enzyme and substrate at low salt concentration. Equation 19 is reminiscent of the classic equation for a pH−rate profile.[53,54] The meaning of each parameter in eq 19 is presented graphically in the notional salt−rate profile of Figure 3.

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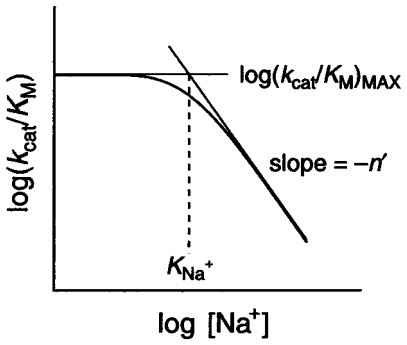

Figure 3. Notional salt−rate profile of enzymatic catalysis. In the profile, $(k_{cat}/K_M)_{MAX}$ is the physical limit of k_{cat}/K_M, K_{Na^+} is the salt concentration at which k_{cat}/K_M is half of its maximal value, and $-n'$ is the slope of the linear region.

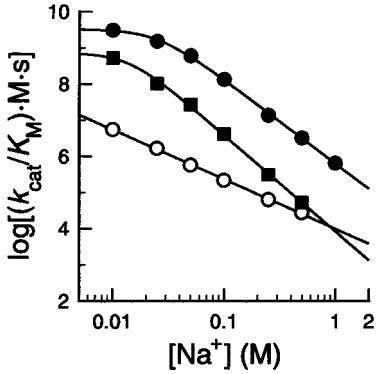

Figure 4. Salt−rate profiles of k_{cat}/K_M for the cleavage of substrate **2** by wild-type ribonuclease A (●) and the K41R (■) and K7A/R10A/K66A (○) variants. Assays were performed at 23 °C in 1.0 mM Bistris−HCl buffer, pH 5.92−6.12. Each curve was fitted to eq 19 (for wild-type and K41R RNase A) or eq 17 (for K7A/R10A/K66A RNase A) by nonlinear regression. Parameters determined from the regression analysis are listed in Table 1.

Salt−Rate Profiles of Wild-Type RNase A and the K7A/R10A/K66A and K41R Variants. The catalytic activity of wild-type RNase A and the K7A/R10A/K66A and K41R variants was measured with fluorogenic substrate **2** in the presence of varying concentrations of NaCl (0.020−1.0 M). Reactions at lower salt concentration reached completion rapidly and were analyzed by using eq 1. Reactions at higher salt concentration occurred slowly and were analyzed by using eq 2.

The effect of salt concentration on catalysis by wild-type RNase A and two variants is depicted in Figure 4. The salt−rate profiles for catalysis by wild-type RNase A and the K41R variant were analyzed with eq 19. Because the value of k_{cat}/K_M for catalysis by K7A/R10A/K66A RNase A did not reach a plateau at low salt concentration, eq 17 was used instead of eq 19. Log(k_{cat}/K_M) rather than k_{cat}/K_M was used as a dependent variable for the regression analysis of salt−rate profiles so as to give more weight to small k_{cat}/K_M values. Nonlinear regression analyses were performed with the program SIGMAPLOT 5.0 (SPSS; Chicago, IL).

Each enzyme loses catalytic activity with increasing salt concentration (Figure 4). Log(k_{cat}/K_M) shows a strong linear correlation with log [Na^+], as proposed in the theoretical model above. The salt−rate profiles of the enzymes do not show any decrease in activity at low salt concentration, as we reported previously for wild-type RNase A.[35] Parameters determined by nonlinear regression analysis are listed in Table 1. Wild-type RNase A and K41R RNase A reach half of their maximal activities at [Na^+] = 25 ± 2 mM and [Na^+] = 14 ± 2 mM, respectively. The $(k_{cat}/K_M)_{MAX}$ value of wild-type RNase A is (3.3 ± 0.4) × 10^9 M^{-1} s^{-1}. The $(k_{cat}/K_M)_{MAX}$ value for K41R RNase A is (0.7 ± 0.2) × 10^9 M^{-1} s^{-1}. The k_{cat}/K_M of K7A/R10A/K66A RNase A does not reach a plateau, even at low salt concentration. The salt−rate profile of each enzyme shows a distinct slope in its linear region. Wild-type RNase A has an n' value of 2.33 ± 0.05, and K41R RNase A has a slightly larger n' value of 2.66 ± 0.08. K7A/R10A/K66A RNase A has a much smaller n' value of 1.37 ± 0.02.

Table 1. Parameters from Salt−Rate Profiles for Cleavage of Substrate **2** by Wild-Type Ribonuclease A and the K7A/R10A/K66A and K41R Variants

	ribonuclease A		
parameter[a]	wild-type	K7A/R10A/K66A	K41R
n'	2.33 ± 0.05	1.37 ± 0.02	2.66 ± 0.08
K_{Na^+} (mM)	25 ± 2		14 ± 2
$(k_{cat}/K_M)_{MAX}$ (10^9 M^{-1} s^{-1})	3.3 ± 0.4		0.7 ± 0.2

[a] Values (±SE) were determined by fitting the data in Figure 4 to eq 19 (wild-type RNaseA and K41R variant) or eq 17 (K7A/R10A/K66A RNaseA) by nonlinear regression.

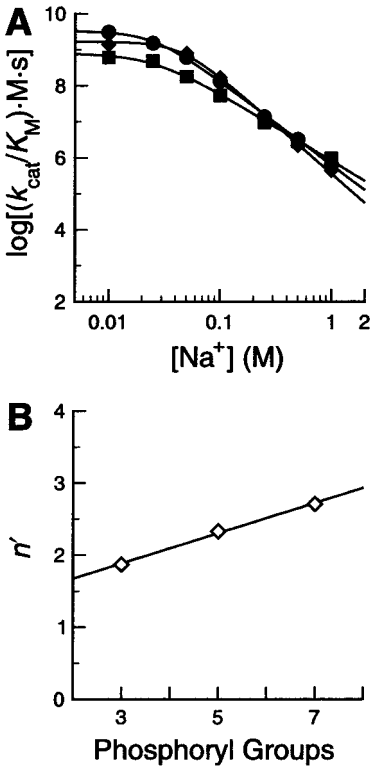

Figure 5. Effect of substrate length on the salt−rate profiles of k_{cat}/K_M for wild-type ribonuclease A. (A) Salt−rate profiles of k_{cat}/K_M for the cleavage of substrate **1** (■), substrate **2** (●), and substrate **3** (◆) in 1.0 mM Bistris−HCl buffer, pH 5.92−6.12. Each curve was fitted to eq 19 by nonlinear regression. Parameters determined from the regression analysis are listed in Table 2. (B) Dependence of n' on the number of phosphoryl groups in substrates. The line has slope = 0.21.

Salt−Rate Profiles with Different Substrates. The effect of salt concentration on the k_{cat}/K_M for cleavage of substrates **1**−**3** by wild-type RNase A is shown in Figure 5A. The parameters from nonlinear regression analysis are listed in Table 2. The n' values of the salt−rate profiles show a monotonic increase in the order 1.87 ± 0.07, 2.33 ± 0.05, and 2.71 ±

Table 2. Parameters from Salt−Rate Profiles for Cleavage of Substrates **1**−**3** by Wild-Type Ribonuclease A

parameter[a]	substrate **1**	substrate **2**	substrate **3**
number of phophoryl groups	3	5	7
n'	1.87 ± 0.07	2.33 ± 0.05	2.71 ± 0.11
K_{Na^+} (mM)	25 ± 5	25 ± 2	44 ± 6
$(k_{cat}/K_M)_{MAX}$ (10^9 M^{-1} s^{-1})	0.8 ± 0.2	3.3 ± 0.4	1.7 ± 0.3

[a] Values (±SE) were determined by fitting the data in Figure 5 to eq 19 by nonlinear regression.

0.11 as the number of phosphoryl groups in substrates **1**−**3** increases in the order 3, 5, and 7, respectively. A plot of n' values versus the number of phosphoryl group in the substrates is shown in Figure 5B and has a linear correlation with a slope of 0.21. Substrate **2** has the highest $(k_{cat}/K_M)_{MAX}$ value, $(3.3 \pm 0.4) \times 10^9$ M^{-1} s^{-1}, and substrate **1** has the lowest $(k_{cat}/K_M)_{MAX}$ value, $(0.8 \pm 0.2) \times 10^9$ M^{-1} s^{-1}. Interestingly, k_{cat}/K_M values for these substrates converge at $[Na^+] \approx 0.4$ M. When $[Na^+]$ is greater than 0.4 M, a longer substrate is a worse substrate. When $[Na^+]$ is less than 0.4 M, a longer substrate is a better substrate. Substrate **3** has the largest K_{Na^+} value, 44 ± 6 mM, and substrates **1** and **2** have the same K_{Na^+} values, 25 ± 5 and 25 ± 2 mM, respectively.

Discussion

Salt concentration, like pH, is a solution condition that can have a dramatic effect on enzymatic catalysis. pH−rate profiles have been used often and with great success to reveal the role of acidic and basic functional groups in catalysis.[55−57] Likewise, salt−rate profiles can provide valuable insight into the role of Coulombic interactions in catalysis, providing that proper care is used in their determination and interpretation.

Salt Effects on the Conformational Stability of Ribonuclease A. Neutral salts can affect the conformational stability of macromolecules.[58−60] Salt effects on the conformational stability of RNase A[61] correlate closely with the Hofmeister series, a rank of the effectiveness of ions in salting-out proteins.[58−60,62] Ions effective in salting-out proteins stabilize the native conformation of RNase A. This salt effect on the conformational stability of the enzyme could introduce a complication in analyzing salt effects on catalysis. NaCl, however, is one of the most inert salts in the Hofmeister series. No decrease to the conformational stability of RNase A has been observed at up to 2 M NaCl.[61] Rather, the addition of NaCl stabilizes RNase A slightly. The temperature (T_m) at the midpoint of the thermal denaturation of RNase A increases monotonically from 59 to 63 °C as the NaCl concentration increases from 0 to 1.0 M.[63] Coulombic repulsion between cationic residues on the surface of RNase A seems to cause a slight decrease in T_m at low salt concentration,[64] as RNase A is a cationic protein (pI = 9.3[65]). This slight change in the conformational stability of RNase A is likely to have a negligible effect on catalysis by the enzyme at ambient temperature.

Effect of Anion Binding to the Active Site. Salt can inhibit catalysis by binding directly to an enzymic active site[66] or by disrupting the local structure of an active site,[67,68] following the order of the Hofmeister series in doing so. Previous work on the salt effect on the stability of an RNase A−nucleic acid complex showed that NaCl and NaF do not differ significantly in their effect on complex stability.[12] Because Cl^- and F^- differ greatly in the Hofmeister series,[58−60,62] this result indicates that anions have a negligible effect on complex stability. A salt−rate profile of k_{cat}/K_M for the cleavage of substrate **2** by RNase A was also determined with NaF to determine the effect of anion type on the catalytic activity of RNase A. The salt−rate profile determined with NaF did not show any statistical difference from that determined with NaCl (data not shown). Hence, anion binding to the active site of RNase A appears to be negligible in the range of salt concentration studied herein (0.01−1.0 M).

Salt Effect on pK_a Values of Active-Site Histidine Residues. Another factor that could affect salt−rate profiles is the salt effect on the pK_a values of active-site residues. According to a pH titration experiment monitored by NMR spectroscopy, the microscopic pK_a values of the active-site histidine residues of RNase A are increased by ∼0.5 unit as the NaCl concentration is increased from 0.018 to 0.142 M.[69] Thus, the salt effect that arises from the perturbation to the pK_a values of the histidine residues is likely to be a negligible component of the observed salt effect on k_{cat}/K_M. Moreover, increasing the salt concentration increases the pK_a value of the Bistris buffer (vide supra), thereby reducing any contribution of the salt effect on the pK_a values of the histidine residues to the salt−rate profiles.

Coulombic Interactions between Ribonuclease A and Its Substrates. The parameter n', which is the absolute value of the slope in a salt−rate profile (Figure 3), reports on the extent of the Coulombic interactions in an enzyme−substrate complex. The n' value, however, is not necessarily identical to n, which is the actual number of counterions released upon formation of the enzyme−substrate complex. The n' value would be identical to n only if the chemistry step limits the value of k_{cat}/K_M (i.e., $C_f \approx 0$) or if $\beta = 1$. Otherwise, n' varies from βn to n.

By applying eqs 17 and 19 to salt−rate profiles, n' values were determined to be 2.33 ± 0.05 and 2.66 ± 0.08 for wild-type RNase A and the K41R variant, respectively. Because K41R RNase A is a sluggish enzyme,[21,24] the value of k_{cat}/K_M for this variant is limited by chemistry. The value of n' for K41R RNase A is therefore similar to the value of n. Because a lysine-to-arginine substitution does not affect the formal charge of the enzyme at neutral pH, wild-type RNase A and the K41R variant manifest the same number of Coulombic interactions. Hence, wild-type RNase A and the K41R variant can be assumed to have the same n value of 2.66. The smaller n' value of the wild-type enzyme indicates that k_{cat}/K_M of wild-type RNase A is not limited solely by chemistry. If the wild-type enzyme does indeed have $C_f \gg 1$, then $n' \approx \beta n$. The experimental values of $n' = 2.33$ and $n = 2.66$ can then be used to calculate $\beta \approx 0.9$, indicating that thermodynamic changes are almost fully manifested in kinetic changes to the association step(s) of the wild-type enzyme.

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K7A/R10A/K66A RNase A is a variant with a reduced Coulombic component to substrate binding. Lys7, Arg10, and Lys66 are not in the active site and are thus likely to interact with the ground state and the transition state uniformly. Replacing these residues with alanine affects k_{cat}/K_M as well as K_M.[12] The salt−rate profile of K7A/R10A/K66A RNase A shows a decrease in catalytic activity at each salt concentration (Figure 4). Moreover, the lower slope of the salt−rate profile compared to that of wild-type enzyme indicates reduced Coulombic interactions. The n' value of the salt−rate profile of K7A/R10A/K66A RNase A was determined to be 1.37 ± 0.02, which is approximately 1.0 and 1.3 units less than the n' values of wild-type RNase A and the K41R variant, respectively.

The five phosphoryl groups of substrate **2** can occupy all four of the known anion-binding subsites of RNase A [P(−1), P0, P1, and P2]. Still, Record and co-workers reported that RNase A can form about seven ion pairs with polymeric single-stranded DNA.[11] The salt−rate profiles of wild-type RNase A with substrates of different lengths corroborate this report. As the length of a substrate increases, the slope of its salt−rate profile also increases (Table 2 and Figure 5). The effect of increasing the number of phosphoryl groups in the substrates seems to be additive. The n' values for the substrates show a linear dependence on the number of phosphoryl groups, with a slope of $\Delta n'/\Delta$(number of phosphoryl groups) = 0.21 (Figure 5B). A quantitative interpretation of this slope is not meaningful, as the substrates are not long enough to assume a homogeneous counterion density. Moreover, n' is not the same as n for wild-type RNase A. It appears, however, that the increase in Coulombic interactions with the longer substrate is responsible for the increase in the n' values.

Comparison with Thermodynamic Salt Effects on Ligand Binding. The value of n can be determined directly by measuring the value of K_d for an RNase A−nucleic acid complex at varying salt concentrations. Previously, we used fluorescence anisotropy to determine K_d for the complex of RNase A and 6-carboxyfluorescein∼d(AUAA) [6-FAM∼d(AUAA)] at varying salt concentrations.[12] With this ligand, the slopes of a plot of log K_d versus log [Na^+] for wild-type RNase A and the K7A/R10A/K66A variant were 2.3 ± 0.1 and 0.9 ± 0.2, respectively. The validity of the analysis of salt−rate profiles herein can be tested by comparing $\partial \log K_d/\partial \log [Na^+]$ with $\partial \log(k_{cat}/K_M)/\partial \log [Na^+]$. Substrate **2**, which was used for the salt−rate profiles in Figure 4, has five phosphoryl groups, whereas the ligand 6-FAM∼d(AUAA) has four phosphoryl groups. Hence, a direct comparison of $\partial \log K_d/\partial \log [Na^+]$ with $\partial \log(k_{cat}/K_M)/\partial \log [Na^+]$ is not feasible. Introducing the K7A/R10A/K66A substitution, however, should result in an identical decrease in the number of counterions released upon complex formation with both the ligand and the substrate. This substitution decreases the value of $\partial \log K_d/\partial \log [Na^+]$ by 1.4 and that of $\partial \log(k_{cat}/K_M)/\partial \log [Na^+]$ by 1.3.[70] This consistency between the behavior of $\partial \log K_d/\partial \log [Na^+]$ and $\partial \log(k_{cat}/K_M)/\partial \log [Na^+]$ supports the validity of the quantitative analysis of salt−rate profile developed herein.

Limit of Catalysis by Ribonuclease A: $(k_{cat}/K_M)_{MAX}$. Like n', $(k_{cat}/K_M)_{MAX}$ is as an informative parameter. Salt−rate profiles show that the catalytic activity of RNase A is maximal at low salt concentration (Figure 4). The value of $(k_{cat}/K_M)_{MAX}$ for wild-type RNase A with substrate **2** is (3.3 ± 0.4) × 10^9 M^{-1} s^{-1} (Table 1). The existence of a plateau in Figure 4 at low salt concentration indicates that additional favorable Coulombic interactions between the enzyme and substrate cannot further increase the value of k_{cat}/K_M beyond that of $(k_{cat}/K_M)_{MAX}$. Accordingly, the value of $(k_{cat}/K_M)_{MAX}$ is likely to be the rate at which the enzyme and substrate associate by diffusion.[71] Thus, the determination of $(k_{cat}/K_M)_{MAX}$ by the analysis of a salt−rate profile can be a useful means to discern the physical limit of catalysis by an enzyme.

The average value of k_{cat}/K_M from triplicate measurements at 0.010 M NaCl is (2.7 ± 0.5) × 10^9 M^{-1} s^{-1}. Few other enzymes are known to have $k_{cat}/K_M > 10^9$ M^{-1} s^{-1}.[72] Superoxide dismutase catalyzes the disproportionation of superoxide with a k_{cat}/K_M of 3.5 × 10^9 M^{-1} s^{-1}.[73] Acetylcholinesterase catalyzes the cleavage of acetylthiocholine with a k_{cat}/K_M of 1.6 × 10^9 M^{-1} s^{-1}.[74] Both of these k_{cat}/K_M values were determined at a low salt concentration (≤0.010 M), where catalysis by superoxide dismutase and acetylcholinesterase is maximal. Catalysis by these two enzymes is limited by diffusion.

Modulation of Uniform Binding. The rate enhancement achieved by an enzyme relies on the binding energy provided by the enzyme to different states on the reaction pathway. Albery and Knowles divided this binding energy into three classes: uniform binding, differential binding, and catalysis of elementary steps.[40] In uniform binding, an enzyme binds to its substrate, product, and transition state(s) to the same extent. In differential binding, an enzyme distinguishes between its substrate and product, binding to one of them more tightly. Finally, in catalysis of elementary steps, an enzyme distinguishes between ground states and transition states, binding to transition states more tightly. Uniform binding has been considered by others to be a waste of binding energy.[75,76] For an irreversible reaction, however, catalytic efficiency is enhanced by increasing uniform binding until any further increase impedes product release.[42]

The addition of salt can provide a simple means to vary the strength of uniform binding in an enzyme-catalyzed reaction. When salt concentration is varied, the free energy of each bound state (ES complex, transition state for the chemistry step, and EP complex) is changed to a similar extent. Accordingly, the large salt effects shown in Figures 4 and 5 are indicative of the large influence of uniform binding on catalysis by RNase A.

Conclusions. We conclude that the effect of salt concentration on enzymatic catalysis can be interpreted with a quantitative model that reveals the contribution of Coulombic interactions to catalysis. The functional groups that are responsible for these Coulombic interactions can be identified by analyzing salt effects with altered enzymes or substrates. A plateau in the value of k_{cat}/K_M in solutions of low salt concentration can reveal the limit imposed by the diffusive encounter of the enzyme and substrate. A deviation of salt−rate profiles from the model could also provide mechanistic information.[77] Many natural polyelectro-

(70) This latter value is relative to $\partial \log(k_{cat}/K_M)/\partial \log [Na^+]$ for K41R RNase A, which is a more appropriate reference than the wild-type enzyme.

(71) The dependence of k_{cat}/K_M for catalysis by RNase A on pH, medium viscosity, and solvent isotope indicates that $(k_{cat}/K_M)_{MAX}$ is indeed limited by the rate of encounter between enzyme and substrate (C. Park and R. T. Raines, unpublished results).

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lytes, including RNA, DNA, polyphosphate, hyaluronic acid, polysialic acid, and heparin, are substrates for enzymes. For these enzymes (as well as others), the quantitative analysis of salt effects is likely to produce novel insight into the energetics of catalysis.

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Supporting Information Available: Derivation of eq 3 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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