The effect of net charge on the solubility, activity, and stability of ribonuclease Sa

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Abstract

The net charge and isoelectric pH (pI) of a protein depend on the content of ionizable groups and their pK values. Ribonuclease Sa (RNase Sa) is an acidic protein with a pI = 3.5 that contains no Lys residues. By replacing Asp and Glu residues on the surface of RNase Sa with Lys residues, we have created a 3K variant (D1K, D17K, E41K) with a pI = 6.4 and a 5K variant (3K + D25K, E74K) with a pI = 10.2. We show that pI values estimated using pK values based on model compound data can be in error by >1 pH unit, and suggest how the estimation can be improved. For RNase Sa and the 3K and 5K variants, the solubility, activity, and stability have been measured as a function of pH. We find that the pH of minimum solubility varies with the pI of the protein, but that the pH of maximum activity and the pH of maximum stability do not.

Keywords: Ribonuclease Sa; isoelectric pH; net charge; electrostatic interactions; protein solubility; enzyme activity; protein stability

The net charge on a protein at any given pH is determined by the pK values (pKs) of the ionizable groups (Tanford 1962). The net charge on a protein is zero at the isoelectric point (pI), positive at pHs below the pI, and negative at pHs above the pI. Ribonuclease Sa (RNase Sa) has 7 Asp, 5 Glu, 2 His, 0 Lys, and 5 Arg residues. Consequently, the wild-type protein has an excess of acidic residues giving it a pI = 3.5 and a net charge ~−7 at pH 7 (Hebert et al. 1997). We have previously reported studies of the thermodynamics of folding and conformational stability of RNase Sa (Pace et al. 1998) and several mutants (Hebert et al. 1998; Grimsley et al. 1999; Pace et al. 2000). In this study, we have replaced Asp and Glu residues with Lys residues singly and in combination to produce variants with different pIs. The residues selected for replacement, Asp 1, Asp 17, Asp 25, Glu 41, and Glu 74, are shown in Figure 1. All of these residues are well exposed to solvent and do not form ion pairs or hydrogen bonds. We report here the experimentally determined isoelectric points for RNase Sa (0K) and the 2K (D17K + E41K), 3K (2K + D1K), 4K (3K + D25K), and 5K (4K + E74K) variants, and we compare these values to the calculated isoelectric points. The addition of five lysines is expected to raise the pI to above 10 and reverse the net charge from −7 to +3 at pH 7. The ability to predict the pI of a protein is useful for developing methods to purify proteins by ion exchange chromatography (Janson andRyden 1989). We also report the dependence of solubility on pH for RNase Sa and the 3K and 5K variants. Theory predicts that the solubility of a protein will be minimal near the pI (Tanford 1961), and we test this in a system where the minimum possible changes in structure are used to vary the pI. Protein solubility is important in diseases such as Alzheimer's disease (Kaytor andWarren 1999), in the devel-

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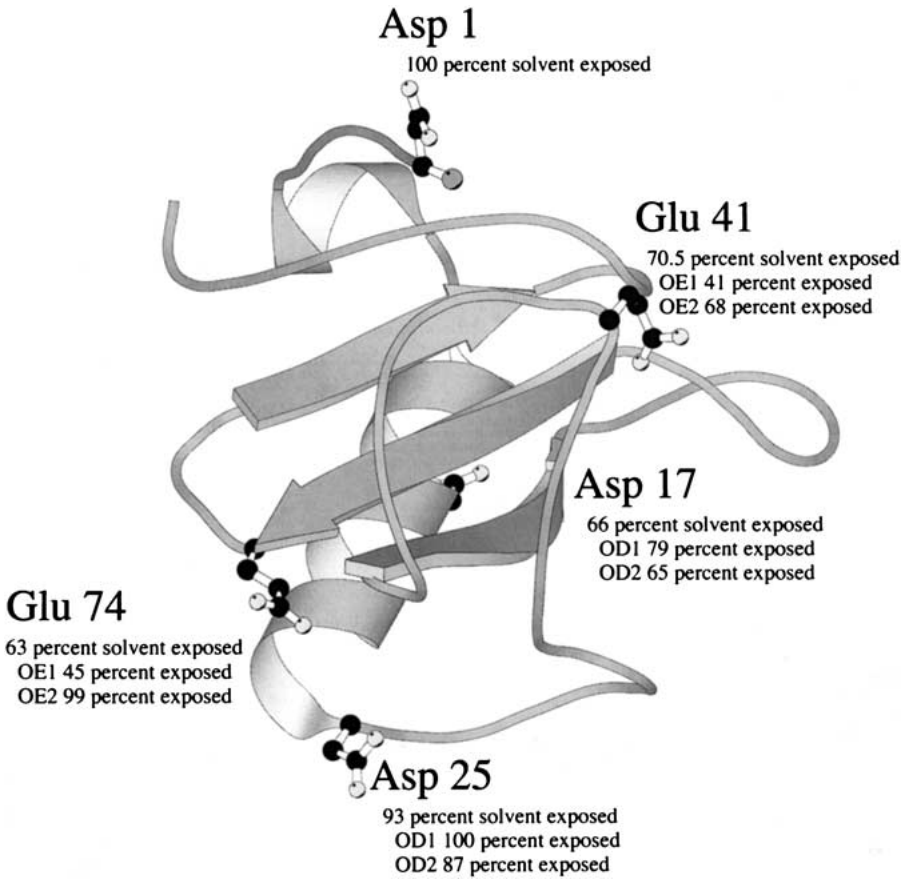

Fig. 1. Ribbon diagram of RNase Sa. The acidic residues changed to lysines are indicated in ball and stick representation. Also given is the percent solvent exposure of the side chain and the oxygens in the carboxyl groups estimated by the method of Lee and Richards (1971). The figure was generated with the program MOLSCRIPT (Kraulis 1991). The PDB identifier for RNase Sa is 1RGG (Sevcik et al. 1996).

opment of recombinant proteins for treating diseases such as fast-acting Lys-Pro insulin (Bakaysa et al. 1996), and to many biochemists, especially X-ray crystallographers (McPherson 1998) and NMR spectroscopists (Harris 1986). We also report the enzyme activity as a function of pH for RNase Sa and the 3K and 5K variants. This is of interest because the natural substrate for the ribonucleases is negatively charged and the net charge on the enzyme might influence the steady-state enzyme kinetics. Finally, we report the pH dependence of the thermodynamics of folding and the conformational stability of RNase Sa and the 3K and 5K variants. The pH dependence of protein stability depends both on the net charge of the protein and on the difference in pKs of the ionizable groups between the folded and unfolded states. Our system may allow us to assess the relative importance of these two effects. There is widespread interest in the pH dependence of protein stability (Tanford 1970; Matthew andRichards 1982; Anderson et al. 1990; Pace et al. 1990; Dao-Pin et al. 1991; Yang andHonig 1993; Antosiewicz et al. 1994; Barrick et al. 1994; Tan et al. 1995; Schaefer et al. 1998; Warwicker 1999; Pace et al. 2000; Whitten andGarcia-Moreno 2000).

Results

The measured and calculated pI values for RNase Sa and the 2K, 3K, 4K, and 5K variants are given in Table 1. The difference between the measured and calculated pI values will be discussed below.

The solubilities of RNase Sa and the 3K and 5K variants are given in Table 2, and the pH dependences are shown in Figure 2. It is clear that the solubility minimum shifts with the pI of the protein from near pH 3.5 for RNase Sa to >pH 9 for the 5K variant. Despite this, the minimum solubility of RNase Sa and the 5K variant is about the same, ~2 mg mL^{-1}. In contrast, the 3K variant is less soluble, having a minimum solubility of 0.5 mg mL^{-1} near pH 7.

The steady-state kinetic parameters K_M and k_{cat} for the hydrolysis of poly inosinic acid (poly(I)) were determined as a function of pH for RNase Sa and the 3K and 5K

Table 1. *Isoelectric points of RNase Sa and the 2K, 3K, 4K, and 5K variants*

Sa variant	Experimental pI[a]	Calculated pI[b]	Net charge at pH 7[c]
Wild type	3.5	4.4 (3.9)	−7
2K	4.6	5.3 (4.8)	−3
3K	6.4	6.6 (6.5)	−1
4K	8.4	8.2 (8.2)	+1
5K	10.2	9.1 (9.1)	+3

[a] pIs of the wild-type and the 2K, 3K, and 4K variants were measured using isoelectric focusing gels and are accurate to ≈0.1 pH units. The pI of the 5K variant was estimated by determining the pH of immobility using continuous polyacrylamide gel electrophoresis and buffer systems recommended by McLellan (1982). This method is accurate to ≈0.3 pH units.

[b] Obtained by calculating the titration curve of the protein using pK values for the amino acid side chains and the α-COOH and α-NH_3^+ termini determined from peptide/model compound studies: Asp = 4.1, Glu = 4.5 (Nozaki and Tanford, 1967); His = 6.6 (McNutt et al. 1990); Tyr = 9.6, Lys = 10.4, Arg = 12.5, α-COOH = 3.6, α-NH_3^+ = 7.8 (Tanford 1968). The values in parentheses were obtained using the average pKs for the side chains of Asp, Glu, and His measured in folded proteins: Asp = 3.4 ± 1.1, Glu = 4.1 ± 0.8 and His = 6.5 ± 0.9. The Asp and Glu values are based on >200 measured pK values in 24 proteins and they were compiled and kindly provided to us by William Forsyth and Andy Robertson at the University of Iowa. The His values are based on 58 measured pK values in 21 proteins and they were compiled and kindly proved by Steve Edgcomb and Kip Murphy at the University of Iowa.

[c] Calculated using the pK values from the model compound data in the footnote above. The net change is rounded to the nearest integer.

variants. The results are given in Table 3. The pH dependence of log (k_{cat}/K_M) is shown in Figure 3. The pH of maximum activity is ~6.5 for RNase Sa and the 3K variant, and ~7.0 for the 5K variant. The 3K variant has somewhat less activity than wild-type RNase Sa, whereas the 5K variant is significantly less active than both. At the pH of optimum activity, (k_{cat}/K_M) is reduced from 1187 (mM sec)$^{-1}$ for wild-type RNase Sa to 105 (mM sec)$^{-1}$ for the 5K variant. The decrease in activity for both variants results entirely from a decrease in k_{cat}.

Table 2. *Solubility as a function of pH for RNase Sa and the 3K and 5K variants*[a]

Wild type		3K		5K	
pH	S (mg mL^{-1})	pH	S (mg mL^{-1})	pH	S (mg mL^{-1})
2.3	11.0	5.4	4.3	6.7	7.8
2.9	3.7	5.7	2.7	6.7	8.5
3.6	2.2	5.9	1.7	7.4	5.9
4.0	2.7	6.5	0.6	7.9	4.6
4.5	4.3	7.3	0.5	8.5	4.1
4.8	7.6	7.7	0.6	8.8	3.2
5.0	9.8	8.3	0.7	9.3	2.3
5.2	17.7	9.3	1.7	10.2	5.3
5.4	23.3				

[a] Measured at 25°C in the presence of 10 mM buffer as described in Materials and Methods.

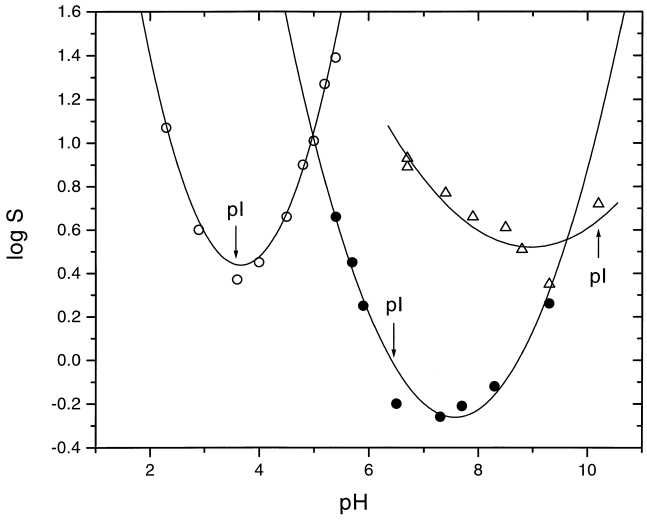

Fig. 2. Solubility of RNase Sa (o) and the 3K (●) and 5K (Δ) variants as a function of pH. The lines have no theoretical significance.

Thermal denaturation curves were determined by monitoring the circular dichroism at 234 nm. The curves for the variants are similar to those previously shown for wild-type RNase Sa (Pace et al. 1998). All curves were analyzed by assuming a two-state mechanism. The analysis yields the melting temperature, T_m, and the van't Hoff enthalpy change at T_m, ΔH_m. These parameters and the differences in conformational stability, $\Delta(\Delta G)$, at pH 7 in 30 mM MOPS are given in Table 4. We also show the calculated $\Delta\Delta G$ values obtained by summing the appropriate $\Delta\Delta G$ values of the single lysine variants. The good agreement between the measured and calculated $\Delta\Delta G$ values shows that the mutational effects are additive.

The pH dependence of the conformational stability of RNase Sa and the 3K and 5K variants was also studied by thermal denaturation. Thermal denaturation curves were determined as a function of pH from pH 2 to pH 10 and analyzed by assuming a two-state mechanism as previously described (Pace et al. 1998). The thermodynamic parameters are summarized in Table 5. The free energy change for folding at any temperature, $\Delta G(T)$, can be calculated using the modified Gibbs-Helmholtz equation,

$$\Delta G(T) = \Delta H_m(1 - T/T_m) + \Delta C_p[(T - T_m) - T\ln(T/T_m)]. \quad (1)$$

We use a value of 1.52 kcal mole^{-1} K^{-1} for ΔC_p in Equation 1 to calculate $\Delta G(25°C)$, based on our earlier studies of the conformational stability of RNase Sa (Pace et al. 1998). Using the results in Table 5 between pH 2 and pH 5, where it is not necessary to correct for the ΔH for buffer ionization, gives $\Delta Cp = 1.31 \pm 0.18$ kcal mole^{-1} K^{-1}. This lower value of ΔC_p would increase the ΔG values by, at most, 4%. Changes in amino acid side chains on the exterior

Table 3. *Kinetic parameters characterizing the cleavage of poly(I) as a function of pH for RNase Sa and the 3K and 5K variants*[a]

	pH	$k_{cat}(s^{-1})$	K_M (mM)	k_{cat}/K_M (mM s)$^-$
Wild type	4.02	2.2	0.25	8.8
	4.47	14.2	0.24	59
	5.05	59	0.21	281
	5.60	131	0.18	728
	6.05	175	0.16	1094
	6.50	178	0.15	1187
	7.06	146	0.19	768
	7.50	95	0.26	365
	8.01	42.1	0.29	145
	8.50	5.6	0.34	16
3K	4.00	1.5	0.75	3.3
	4.50	7.5	0.57	13
	5.00	24	0.33	73
	5.50	70	0.26	269
	6.00	98	0.18	544
	6.50	112	0.14	800
	7.06	88	0.14	629
	7.48	51	0.19	268
	8.00	26	0.20	130
	8.48	8	0.24	33
5K	4.47	0.3	1.10	0.3
	5.04	1.2	0.64	1.9
	5.60	4.7	0.47	10
	6.05	7.7	0.23	34
	6.50	11.1	0.13	85
	7.06	10.5	0.10	105
	7.50	6.7	0.10	67
	8.10	3.5	0.11	32
	8.50	1.3	0.14	9.3

[a] Measured at 25°C in the presence of 0.05 M Tris, 0.1 M potassium chloride and 0.05 M sodium acetate as described in Materials and Methods.

of a protein are expected to have only a small effect on ΔC_p because the side chains will be largely exposed to solvent in both the folded and unfolded conformations. Therefore we decided to use the more accurately determined value of 1.52 kcal mole^{-1} K^{-1}. Note that all three proteins are maximally stable near pH 5, with stabilities (kcal mole^{-1}) of 7.6 for RNase Sa, 5.4 for 3K RNase Sa, and 5.6 for 5K RNase Sa.

Discussion

Isoelectric points of RNase Sa and the charge reversal variants

Knowing the pI of a protein is helpful to protein chemists developing purification schemes or performing experiments in which the protein's solubility is a crucial factor. (As discussed below, proteins tend to be the least soluble near their pI.) The pI is usually determined experimentally by isoelectric focusing (Righetti et al. 1981); however, one can estimate a protein's pI by calculation if the amino acid

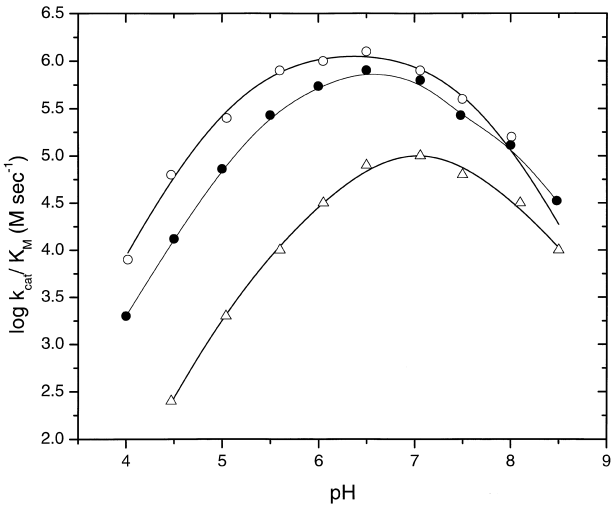

Fig. 3. pH dependence of log (k_{cat}/K_M) for the cleavage of poly(I) by RNase Sa (o) and the 3K (●) and 5K (Δ) variants.

composition and the pKs of the ionizable groups are known. Usually the pKs of the groups are not known, so values obtained from model compound studies are used for the calculation. Because these pKs may differ significantly from the pKs of groups in the folded protein, calculated pIs often disagree with experimentally measured pIs. A number of methods have been proposed for the theoretical determination of the pIs of proteins (Sillero andRibeiro 1989; Patrickios andYamasaki 1995). Typically, these methods give results that are within ±1 pH unit of the experimental pI.

In Table 1 we give the experimentally determined pIs for RNase Sa and the 2K, 3K, 4K, and 5K variants. The value of 3.5 obtained for RNase Sa is in exact agreement with our previously reported value (Hebert et al. 1997). Thus, RNase Sa is an acidic ribonuclease like RNase T1, which has a pI of 3.8 (Hebert et al. 1997). In contrast, barnase, another well-studied microbial ribonuclease, is a basic protein with a pI of 9.2 (Bastyns et al. 1996). Replacing three acidic residues with lysines (3K) raises the pI close to neutrality, and replacing five acidic residues with lysines (5K) raises the pI above 10. By reversing five charges on RNase Sa, we have changed it from one of the most acidic proteins to one of the most basic proteins. This is shown in Table 6, where we give the experimentally determined pIs and calculated pIs for a number of well-studied proteins.

When the pIs are calculated for RNase Sa and the 3K and 5K variants using pK values taken from model compound studies (Table 1), the pI is overestimated for RNase Sa and the 2K and 3K variants and underestimated for the 4K and 5K variants. When the calculation is performed using the average pKs for Asp, Glu, and His measured in folded proteins, the agreement between measured and calculated improves for RNase Sa and the 2K and 3K variants. The pKs of the ionizable groups of RNase Sa have been measured by

Table 4. *Parameters characterizing the thermal unfolding of RNase Sa and the charge reversal variants at pH 7*[a]

Sa variant	T_m[b] (°C)	ΔH_m[c] (kcal mole^{-1})	$\Delta\Delta G$[d] (kcal mole^{-1})	$\Delta\Delta G$[e] (kcal mole^{-1})
Wild type	47.2	91	—	—
D1K	48.7	89	0.4	—
D17K	43.3	90	–1.1	—
D25K	50.2	93	0.9	—
E41K	42.9	92	–1.2	—
E74K	51.1	94	1.1	—
2K (E41K,D17K)	39.5	83	–2.2	–2.3
3K (E41K,D17K,D1K)	40.4	78	–1.9	–1.9
4K (E41K,D17K,D1K,D25K)	44.4	84	–0.8	–1.0
5K (E41K,D17K,D1K,D25K,E74K)	46.8	82	–0.1	0.1

[a] pH 7, 30 mM MOPS.
[b] Midpoint of the thermal unfolding curve. The standard deviation is ±0.3°C.
[c] Enthalpy change at T_m. The standard deviation is ±5%.
[d] $\Delta\Delta G_{meas} = \Delta T_m \times \Delta S_m$ (wild type). See Becktel and Schellman (1987) or Pace and Scholtz (1997) for a discussion of this method. A positive sign indicates an increase in stability.
[e] Calculated by summing the appropriate $\Delta\Delta G$ values of the single mutant variants.

NMR (D. Laurents, pers. comm. Similar measurements on the 5K variant are in progress.) When these values are used in the calculation, the pI = 3.7, in good agreement with the measured value. Similarly, if the pK values measured for RNase Sa are used for the 5K variant, the calculated pI = 10.1, again in excellent agreement with the measured pI. The calculated pI for the 5K variant in Table 1 is low because RNase Sa has eight Tyr that are largely buried and they all have pKs >11. This is much higher than the pK = 9.6 that is assumed for tyrosine -OH groups on the basis of model compound studies. This may be true with other proteins because Tyr side chains are 76% buried on average in a large sample of folded proteins (Lesser and Rose 1990).

The fact that the pKs of the ionizable groups in model compounds may differ significantly from the pKs of the same groups in folded proteins is reflected by the calculated and measured pI values shown in Table 6. In general, acidic proteins have their pIs overestimated and basic proteins have their pIs underestimated when calculated using pKs from model compound studies. This is expected. For Asp side chains, a pK = 4.1 is observed with uncharged model compounds, but a pK = 3.4 is the average value observed in proteins (See the footnotes to Table 1). This results mainly because proteins have a net positive charge in the region where Asp and Glu carboxyls titrate, and this will tend to lower their pKs relative to those measured in uncharged model peptides (Antosiewicz et al. 1996). The converse will be true for basic proteins, where the proteins will have a net negative charge above the pI that will tend to raise the pKs of the ionizable groups. In addition to net charge, other environmental effects can change pKs. For example, we have shown that the completely buried and hydrogen bonded carboxyl of Asp 76 in RNase T1 has a pK

Table 5. *Parameters characterizing the thermal unfolding of RNase Sa and the 3K and 5K variants between pH 2 and 10*

Sa variant	pH	T_m[a] (°C)	ΔH_m[b] (kcal mole^{-1})	ΔS_m[c] (cal K^{-1} mole^{-1})	ΔG (25°C)[d] (kcal mole^{-1})
Wild type	2	27.3	56	186	0.42
	3.3	42	85	270	3.88
	5	54.2	108	330	7.59
	7	47.2	91	284	5.11
	8.3	39.5	92	294	3.75
	9	35.4	78	253	2.36
	10	30.1	72	237	1.15
3K	2	27.9	60	199	0.56
	3.3	44.4	83	261	4.15
	4	50.8	89	275	5.48
	5	49.4	91	282	5.44
	6	50.0	84	260	4.99
	7	40.7	77	245	3.24
	8.3	31.9	70	230	1.46
	9	29.1	62	205	0.80
	10	22.3	50	169	–0.48
5K	2	25.6	62	208	0.12
	3	41.4	77	245	3.31
	4	52.4	85	261	5.35
	5	53.3	87	267	5.62
	6	51.3	85	262	5.22
	7	47.3	82	256	4.50
	8	44.2	76	240	3.70
	9	42.7	72	228	3.50
	10	36.4	59	191	1.85

[a] Midpoint of the thermal unfolding curve. The standard deviation is ±0.3°C.
[b] Enthalpy change at T_m. The standard deviation is ±5%.
[c] $\Delta S_m = \Delta H_m/T_m$.
[d] Calculated with Equation 1 using the T_m and ΔH_m values in this table and a value of 1.52 kcal K^{-1} mole^{-1} for ΔCp (Pace et al. 1998).

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