ASP 79 MAKES A LARGE, UNFAVORABLE CONTRIBUTION TO THE CONFORMATIONAL STABILITY OF RIBONUCLEASE SA

A Senior Honors Thesis

by

SAUL RENE TREVINO

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

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RESEARCH FELLOWS

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Approved as to style and content by:

C. N. Pace
C. Nick Pace
(Fellows Advisor)

Edward A. Funkhouser
Edward A. Funkhouser
(Executive Director)

April 2001

Group: Biochemistry

ABSTRACT

Asp 79 Makes a Large, Unfavorable Contribution to the Stability of Ribonuclease Sa. (April 2001)

Saul Trevino
Department of Biochemistry/Biophysics
Texas A&M University

Fellows Advisor: Dr. C. Nick Pace
Department of Medical Biochemistry and Genetics

An important unsolved problem in the field of biochemistry is the protein folding problem. Extensive research is being done to predict the structure and function of a protein given just its linear sequence of amino acids. The answer to this problem will lie in understanding the interactions involved in protein folding.

Ribonuclease Sa (RNase Sa) is a relatively small protein with just 96 amino acids, and it is a good model for studying protein folding. The side-chain carboxyl of Asp 79 in RNase Sa is 89% buried, partially charged, and does not form any intramolecular hydrogen bonds. This amino acid was replaced by several amino acids including hydrophobic, polar uncharged, and positively charged amino acids, and the stability of the protein increased in each substitution that was introduced. The range of stability increase was from 1.7 to 2.9 kcal/mol. Thus, Asp 79 contributes unfavorably to the stability of the protein, and it is a good site for mutation in order to increase the

stability of the protein. Also, the stability was measured as a function of pH for RNase Sa and the D79F mutant. The pK of Asp 79 was found to be 7.2 in the native state and 4.7 in the denatured state.

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I would like to thank Dr. Nick Pace for all of his help and guidance throughout my college years. I would also like to thank Dr. Marty Scholtz, Dr. Kevin Shaw, Stephanie Newsom, David Schell, Richard Thurlkill, Dr. Gerald Grimsley, Dr. Beatrice Huyghues-Despointes, and Roy Alston for all of their help with this project.

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INTRODUCTION

Proteins carry out many of the important and difficult tasks in all living organisms. Proteins must fold the right way in order to function properly. Thus, the most important reaction in living organisms may be protein folding, where the protein goes from the unfolded form to the folded form.

Extensive research has been done on what is called the protein folding problem. Essentially, this deals with being able to predict the shape and function of a protein given its linear sequence of amino acids. However, protein folding is not an easy process to understand because there are many interactions to consider such as hydrophobic interactions, electrostatic interactions, hydrogen bonding, and conformational entropy to name a few. Levinthal's paradox states that if a small protein of 100 residues sampled every possible conformation available to it on the way to the native (folded) state, the folding time for a protein would be longer than the age of the universe (1). Therefore, there are so many possible shapes a protein can take that if it tested each possible shape, the protein would take forever to fold. This makes it hard to predict the particular shape of a protein since there are so many possibilities to choose.

Understanding the way proteins fold is important because it has many medical implications. For example, mad cow disease is a disease that is caused by the misfolding of prion proteins in the brain. In this disease, the prion proteins are mutated and fold in a different way. These misfolded prion proteins can cause other prion proteins in the brain

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This thesis follows the style and format of *Biochemistry*.

to misfold and aggregate, and this is what causes mad cow disease. If it could be understood what is causing these proteins to fold improperly and how to get them to fold the right way, then this could lead to a cure for mad cow disease. Improper protein folding is also the cause of Alzheimer's disease and cystic fibrosis.

The main topic of this research has been understanding how the conformational stability of a protein can be increased. The conformational stability of a protein can be defined as the free energy difference between the native and the denatured states of the protein (2). For many industrial applications, the most stable protein is desired which still has the desired enzyme activity.

The protein used in this study is RNase Sa. RNase Sa is a relatively small protein with just 96 amino acids, and it is a good model for studying protein folding. This protein has three aspartic acid residues that are buried. Two of these three aspartic acid residues form intramolecular hydrogen bonds. The other buried aspartic acid at position 79 does not make any ion pairs or intramolecular hydrogen bonds. The approach taken to increase the stability of this protein was to mutate the aspartic acid at position 79 (Asp 79) to other amino acids. This amino acid was chosen because it is negatively charged meaning that one would expect to find it on the surface of the protein. However, this amino acid is 89% buried which means that most of it resides in the interior of the protein where one would only expect hydrophobic amino acids to be found. In general, it is unfavorable for a protein to have a charged amino acid in the hydrophobic interior of the protein if it does not form any ion pairs or intramolecular hydrogen bonds. Therefore, the initial strategy to increase the stability of the protein

was to change this charged amino acid to a hydrophobic amino acid. Also, ion pairs and hydrogen bonds have been shown to be stabilizing forces in protein folding (3,4). Therefore, no stability is lost in making these mutations due to removing any ion pair or hydrogen bond interactions. Several mutations were made at this position, and the change in stability of each mutant was determined by using circular dichroism spectroscopy to measure thermal denaturation curves.

The pK_a of Asp 79 was also determined. The conformational stability of most proteins is dependent on pH. Ionizable groups such as Asp 79 contribute to the pH dependence of these proteins. The pH dependence of the conformational stability of a protein can be determined by considering the differences in the titration curves of the folded and unfolded proteins. The pH dependence of the conformational stability of RNase Sa was used to determine the pKa of Asp 79 according to the method described by Quirk et al (5).

MATERIALS AND METHODS

Making the mutants Several mutants were made for this project. In making a mutant, the first step is to design some mutagenic primers. The mutagenesis kit that was used is called Quickchange which is produced by Stratagene. In the Quickchange protocol some criteria for primer design are given, and the primers were designed according to these criteria.

After designing the primers according to the criteria, the next step was to order the primers. The primers and their compliments were ordered from Integrated DNA Technologies at www.idtdna.com. When the primers arrived, the DNA was dissolved in 100 µl of sterile distilled water. From these concentrated DNA solutions, dilute solutions of the primers were made at 5pmol/µl.

These 5 pmol/µl primers were then used in the Quickchange reactions. The reactions were prepared as suggested in the Quickchange protocol. These reactions were then put into a Perkin Elmer GeneAmp PCR System 2400 thermocycler. The reactions were held once at 95°C for 30 seconds. Then the reactions went through 18 cycles of 95°C for 30 seconds → 55°C for 1 minute → 68°C for 14 minutes. Then the reactions were held at 4°C until the next step where 1 µl of an enzyme called Dpn 1 was added to the reactions, and then the reactions were held at 37oC for 1 hour in the thermocycler.

The next step was to transform the Quickchange reactions into E. Coli XL1-Blue supercompetent cells. The reactions were transformed according to the directions in the Quickchange protocol. The transformations were plated on LB agar plates containing ampicillin at a concentration of 50 µg/ml.

After the transformations were incubated for more than 16 hours, they were removed from the incubator. One of the colonies from the agar plate was then placed into a culture tube containing 5 ml of LB media along with 5 µl of a stock solution of ampicillin which was at a concentration of 50 mg/ml. The culture tube was then incubated overnight at 37°C with shaking.

After these overnight cultures were incubated overnight, they were removed from the incubator and the cells were resuspended in the media by vortexing. Then the plasmid DNA was removed from the cells by using the QIAprep Spin Miniprep Kit which is produced by Qiagen. After the minipreps were done, about 50 µl of mutant plasmid DNA was obtained. About 20 µl of this DNA was aliquoted into another eppendorf tube and dried in a speed vacuum apparatus. When the tube was dry, the DNA was dissolved in 5 µl of sterile distilled water, and the DNA was sent to be sequenced at the Gene Technologies Laboratory (Department of Biology, Texas A&M University). The sequencing results were then checked to make sure that the proper mutation was made.

Protein expression and purification Wild-type RNase Sa was expressed and purified according to Hebert et al (6). As for the D79 mutants of RNase Sa, once the plasmid with the mutant DNA was made, the DNA was transformed into *E. Coli* MQ competent cells and plated on LB agar plates containing ampicillin at a concentration of 50 µg/ml. The plates were then incubated at 37°C overnight. Six liters of Terrific Broth was prepared and separated into twelve 2L Erlenmeyer flasks in 500 ml aliquots. This media

was then autoclaved. Twelve milliliters of ampicillin at a concentration of 50 mg/ml and 6 milliliters of IPTG at a concentration of 0.1 M were prepared as well. Once the plates were incubated overnight, one of the colonies was picked and used to inoculate a 60 ml LB culture which contained ampicillin at a concentration of 50 μg/ml. This culture was then incubated with shaking in a 37°C water bath. About four hours later, when the LB culture showed cell growth, the twelve 2L flasks containing the Terrific Broth media were then inoculated with the 60 ml LB culture. To each flask, 4 ml of the 60 ml LB culture and 500 μl of ampicillin at a concentration of 50 mg/ml was added. These flasks were then incubated at 37oC with shaking. When the optical density of the media in the flasks reached about 0.6, protein expression was induced by adding 500 μl of 0.1 M IPTG to each flask. 500 μl of ampicillin at a concentration of 50 mg/ml was also added to each flask at this point. The flasks were then incubated at 30°C with shaking. Four hours after induction, the cells were harvested by centrifugation, and the proteins in the periplasm were removed from the cells according to the method described by Hebert et al (6). In order to precipitate some of the unwanted proteins, the periplasmic fraction (about 2 L) was warmed to room temperature, and sodium succinate and succinic acid was added to bring the periplasmic fraction to 50 mM succinate/20 mM Na^+. Then the pH of the periplasmic fraction was adjusted to 3.25 with concentrated hydrochloric acid. The proteins that precipitated were removed by centrifugation. The periplasmic fraction was then loaded onto an SP-Sephadex cation exchange column, and the column was then washed with 500 ml of 50 mM Succinate/20 mM Na^+ buffer at pH 3.25. The protein on the column was then eluted with a 1 L linear gradient of 50 mM Succinate/20 mM Na^+

buffer at pH 3.25 to 50 mM Succinate/100 mM Na^+ buffer at pH 6.0. Fractions of the column eluent were collected, and then an activity assay was performed according to the method described by Hebert et al (6) in order to determine which fractions contained ribonuclease activity. These active fractions were then pooled and freeze dried. The freeze dried sample was then dissolved in less than 50 ml of 50 mM ammonium bicarbonate buffer and applied to a Sephadex G-50 column as described by Hebert et al (6). The G-50 column separated the proteins by size and fractions were collected. The fractions containing ribonuclease activity were then pooled and freeze dried. Then the purified protein was labeled and stored at –20°C.

Experimental buffers All buffers used for thermal denaturations were 30 mM in concentration. The following buffers were used: pH 2 to 3.5 and pH 9 to 10, glycine, pH 3.5 to 5.5, acetate, pH 5.5 to 6.5, MES, pH 6.5 to 8.5, MOPS.

Thermal Denaturations Once the mutant proteins were expressed and purified, thermal denaturation experiments were done using an AVIV Circular Dichroism Spectrometer Model 62DS. About 1 mg of purified protein was added to an eppendorf tube and dissolved in 1 ml of the appropriate buffer (e.g. 30 mM MOPS buffer at pH 7.0). A 1:10 dilution was then made by taking 100 µl of the dissolved protein and adding 900 µl of sterile distilled water. Then an absorbance measurement of the 1:10 dilution was taken at 278 nm using a Hewlett Packard 8452A Diode Array Spectrophotometer. Knowing that a 0.1 mg/ml solution of protein gives an absorbance of 0.12 at 278 nm, a proportion

was used to determine the concentration of protein in the 1:10 dilution. This concentration was then multiplied by 10 to obtain the protein concentration of the original dissolved protein solution. The cuvettes for the circular dichroism spectrometer hold 2.4 ml and the desired concentration of the protein in 30 mM buffer was 0.1 mg/ml. After preparing the 0.1 mg/ml protein samples in 30 mM buffer, the sample was then placed in the spectrometer and the following settings were used to perform the thermal denaturation experiment:

Averaging Time = 30.0 seconds

Equilibration Time = 3.0 minutes

Wavelength = 234.0 nm

Temperature Range = 5°C to 90°C by 1°C

The instrument measured the loss of positive ellipticity at 234 nm. After the program finished, the data was saved and then analyzed using Microcal Origin version 6.0 (Microcal Software, Inc, Northhampton, MA).

Analysis of the data using Microcal Origin involved fitting the data using non-linear regression and the equation:

$$y = ((YN+MN*x)+(YD+MD*x)*\exp(-dH*(1/(x+273.15)-1/(Tm+273.15))/0.001987))/(1+\exp(-dH*(1/(x+273.15)-1/(Tm+273.15))/0.001987)) \quad (1)$$

according to the method of Santoro and Bolen (7). YN is the y-intercept of the pre-transition baseline, MN is the slope of the pre-transition baseline, YD is the y-intercept of the post-transition baseline, MD is the slope of the post-transition baseline, dH is the enthalpy change associated with the unfolding reaction, and Tm is the melting

temperature of the protein. Fitting the data using eq 1, gives the important thermodynamic parameters ΔH_m and T_m.

Once T_m for the mutant is obtained, the change in stability of the mutant, $\Delta(\Delta G)$, can be determined according to Becktel & Schellman using the equation (8):

$$\Delta(\Delta G) = \Delta T_m * [\Delta H_m(wt)/T_m(wt)] \quad (2)$$

where ΔT_m is the difference in melting temperatures between the mutant and the wild-type protein ($T_{m,mutant} - T_{m,wild\text{-}type}$). A positive $\Delta(\Delta G)$ value indicates that the mutant protein is more stable than the wild-type protein. $\Delta(\Delta G)$ values were obtained for all D79 mutants, and for the D79F mutant at various pHs.

$\Delta(\Delta G)$ values were also obtained for the D79F mutant at various pHs to determine the pK_a of the aspartic acid at position 79. The $\Delta(\Delta G)$ values were then plotted versus pH, and the resulting curve was fit to the following equation (5):

$$\Delta(\Delta G) = \Delta(\Delta G)([H^+] = \infty) - RT\ln([1+K_{i,N}/[H+]]/[1+K_{i,D}/[H+]]) \quad (3)$$

RESULTS

Determination of the stability of wild-type RNase Sa and the Asp 79 mutants at pH 7 A typical thermal denaturation curve is shown in Figure 1. From this curve, the melting temperature of the protein (T_m) and the enthalpy change of the unfolding reaction (ΔH_m) can be determined. The T_m is the midpoint of the transition region, and ΔH_m is the enthalpy change for unfolding at T_m (9). These thermodynamic parameters for wild-type RNase Sa and the Asp 79 mutants as determined by fitting the data from the thermal denaturation curves to eq. 1, are given in Table 1. The $\Delta(\Delta G)$ values as determined by using eq. 2 are also given in Table 1. The results for wild type Sa agree with previous results (9). An attempt was made to purify D79A, but it was not successful. Hence, there are no data for D79A in Table 1.

Determination of the pK_a of Asp 79 Thermal denaturation experiments on D79F were run at pH values ranging from 2.00 to 10.00. Table 2 shows the thermodynamic results from these experiments. Figure 2 shows the pH dependence of $\Delta(\Delta G)$.

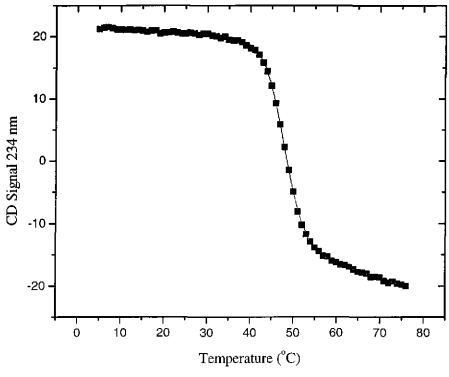

Figure 1: A thermal denaturation curve of RNase Sa in 30 mM MOPS buffer at pH 7.0. The solid line is obtained by fitting eq 1 to the data.

Table 1: Parameters Characterizing the Thermal Unfolding of RNase Sa and the D79 Mutants at pH 7.0[a]

Protein	ΔH_m (kcal/mol)	T_m (°C)	$\Delta(\Delta G)$[b] (kcal/mol)
RNase Sa	91.1	47.8	
D79F	89.0	57.8	3.0
D79Y	83.5	57.4	2.9
D79L	75.5	56.6	2.6
D79I	89.1	57.4	2.7
D79K	87.9	55.4	2.3
D79N	96.2	53.4	1.7
D79A	-	-	-

[a]The thermal denaturation curves were determined in 30 mM MOPS buffer, at pH 7. [b]The values of ΔH_m and T_m for RNase Sa and the mutants were used in eq 2 to calculate the $\Delta(\Delta G)$ values. The error in ΔH_m was $\pm$ 5%, the error in T_m was $\pm$ 0.3, and the error in $\Delta(\Delta G)$ was $\pm$ 0.2.

Table 2: Parameters Characterizing the Thermal Unfolding of RNase Sa and D79F at Various pH values[a]

Protein	ΔH_m (kcal/mol)	T_m (°C)	ΔS_m (cal $K^{-1}mol^{-1}$)	$\Delta(\Delta G)^b$ (kcal/mol)	Error in $\Delta(\Delta G)$
wt pH 2.0	53.8	26.1	180		
D79F pH 2.0	43.3	27.0		0.2	0.0
wt pH 3.06	85.9	39.0	275		
D79F pH 3.06	67.5	38.5		-0.1	-0.1
wt pH 4.0	101.0	49.1	314		
D79F pH 3.93	88.2	49.9		0.2	0.0
wt pH 5.0	97.4	54.1	298		
D79F pH 5.0	76.8	58.6		1.3	0.0
wt pH 5.5	90.2	52.8	277		
D79F pH 5.5	77.2	57.9		1.4	0.1
wt pH 6.0	92.7	51.2	286		
D79F pH 6.0	78.3	58.5		2.1	0.0
wt pH 6.5	93.2	49.2	289		
D79F pH 6.5	87.0	58.5		2.7	0.1
wt pH 7.0	89.8	47.7	280		
D79F pH 7.0	87.0	58.1		2.9	0.1
wt pH 7.5	101.2	44.2	319		
D79F pH 7.5	69.1	55.4		3.6	0.1
wt pH 8.0	86.4	41.6	275		
D79F pH 8.0	86.9	54.1		3.4	0.2
wt pH 8.5	86.7	39.0	278		
D79F pH 8.5	81.6	51.8		3.6	0.1
wt pH 9.5	72.1	32.3	236		
D79F pH 9.5	82.2	49.5		4.1	0.3
wt pH 10.0	71.6	31.2	235		
D79F pH 10.0	80.7	49.9		4.4	0.1

[a]The thermal denaturation curves were determined at a 30 mM concentration of the appropriate buffer. [b]The values of ΔH_m and T_m for wild-type and the mutant were used in eq 2 to calculate the $\Delta(\Delta G)$ values.

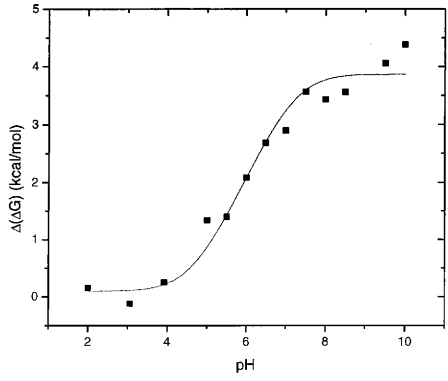

Figure 2: $\Delta(\Delta G)$ vs pH. The solid line was obtained by fitting eq 3 to the data between pH 2.00 and 10.00. The fitted parameters obtained were $pK_{i,N} = 7.2 \pm 0.2$, $pK_{i,D} = 4.7 \pm 0.2$, and $\Delta(\Delta G)([H^+] = \infty) = 0.1 \pm 0.2$.

CONCLUSION

Contribution of Asp 79 to RNase Sa Stability Figure 3 shows where Asp 79 is in the protein. Since most of this negatively charged residue resides in the hydrophobic interior of the protein, it was thought that this would be unfavorable for the stability of the protein. Therefore, the initial strategy was to mutate the aspartic acid to a more hydrophobic amino acid like phenylalanine. This resulted in an increase in stability of about 2.9 kcal/mol. This was the highest increase in stability for RNase Sa that had been seen with a single mutation.

This led us to try other mutations at this position to see how they affected the stability of the protein. For example, we mutated the aspartic acid to other hydrophobic amino acids (leucine, isoleucine), to polar, uncharged amino acids (tyrosine, asparagines), and even a positively charged residue (lysine). All of the mutations gave increases in stability. Comparing the phenyalanine, leucine, and isoleucine mutations, there was no direct relation between the hydrophobicity of the residue that was substituted and the increase in stability that the mutation gave (see Table 3). However, there seemed to be a direct relation between the side chain volume and the increase in stability that the mutation gave. This was interesting considering that in a paper by Yutani et al., 1997 (15), they saw a linear increase in stability with increasing hydrophobicity of the substituted amino acid at position 49 of tryptophan synthase α subunit, unless the volume of the substituted amino acid was over a certain limit. At

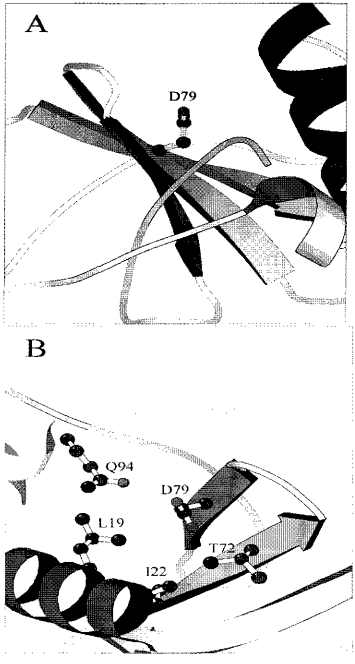

Figure 3: (A) Ribbon diagram of RNase Sa showing the side chain of Asp 79. (B) A top view of the side chain of Asp 79 from its orientation in panel A to show the four amino acids that surround Asp 79: Gln 94, Leu 19, Ile 22, and Thr 72. The figure was created with *MOLSCRIPT* (*10*) based on the 1.2 Å crystal structure (*11*).

Table 3: Hydrophobicities, Side Chain Volumes, and Δ(ΔG) Values for the D79L, D79I, and D79F Mutations

Mutation at Position 79	Hydrophobicity Profile[a]	Hydrophathy Index[b]	Side-chain Volume ($Å^3$)[c]	Δ(ΔG) (kcal/mol)
Leucine	-1.16	3.8	100.8	2.6
Isoleucine	-1.02	4.5	101.1	2.7
Phenylalanine	-1.36	2.8	129.7	3.0

[a]The hydrophobicity profile for the indicated residue at position 79 in RNase Sa was calculated according to the program at http://grserv.med.jhmi.edu/~raj/misc/hpphob2.html (12) with the following settings: Window size = 5, Hydrophobicity Scale = Kyte and Doolittle, Raw Data, Sequence = RNase Sa. [b]The hydropathy index values were obtained from Kyte and Doolittle (13). [c]The side-chain volumes were obtained from Harpaz et al (14).

position 79 in RNase Sa, there does not appear to be a limit to the volume that can be added to this position without sacrificing the increase in stability.

The mutations to the polar, uncharged residues tyrosine and asparagines also gave considerable increases in stability. The mutation to tyrosine gave an increase in stability that was just as high as the mutation to phenylalanine (about 2.9 kcal/mol). Considering that tyrosine is a polar, uncharged residue, one would not expect such a high increase in stability with this mutation since a polar residue is being placed in the hydrophobic interior. The polar region of tyrosine is the hydroxyl group at the end of the phenyl ring so the hydroxyl group could possibly reach far enough to be near the surface of the protein, whereas the hydrophobic region of the amino acid (the phenyl ring) would be positioned in a similar way as the phenyl ring in the phenylalanine mutation giving rise to favorable hydrophobic interactions in the hydrophobic interior of the protein. The mutation to asparagine also gave an increase in stability (about 1.7 kcal/mol), but it was the smallest increase in stability of the mutations that were done. The polar region of asparagine is also located at the end of the amino acid, but since asparagine is not as long as tyrosine, the polar region probably does not reach to the surface of the protein and is therefore in the hydrophobic region of the protein. This result suggests that while it is not as favorable to have a polar, uncharged region in the hydrophobic interior of the protein as it is to have a hydrophobic region, it is more favorable than having a charged region in the interior of the protein.

Surprisingly, the mutation to the positively charged amino acid, lysine, gave an increase in stability that was comparable to the mutation to leucine. This result is

interesting because inserting a charged amino acid gave almost as high of an increase in stability as inserting a hydrophobic amino acid. In a similar fashion as the mutation to tyrosine, this could possibly be explained by considering that the charged group of lysine is at the end of the amino acid, and since lysine is longer than aspartic acid, the charged group could reach far enough to be on the surface of the protein. Also, the hydrophobic portion of the amino acid (the four methylene groups) would be buried favorably in the interior of the protein.

There are no data for D79A in Table 1. This is because we were unable to express and purify this protein. It has been shown in a paper by Matthews (16), that cavity creating mutations such as mutating an aspartic acid to an alanine can be destabilizing to a protein. Therefore, D79A was probably too unstable to express and purify.

pK_a of Asp 79 The pK_a of Asp 79 was determined according to the method described by Quirk et al (5). When eq 3 was fit to the stability data as a function of pH, it was found that $pK_{i,N} = 7.2 \pm 0.2$, $pK_{i,D} = 4.7 \pm 0.2$, and $\Delta(\Delta G)([H^+] = \infty) = 0.1 \pm 0.2$ (see Figure 2). Based on these results, it is estimated that the pK of Asp 79 is 7.2 ± 0.2 in native and 4.7 ± 0.2 in denatured RNase Sa. Considering that the normal pK of an aspartic acid residue is around 4, the pK of Asp 79 is high. This is expected since it is 89% buried and probably resists ionization.

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VITA

Saul Rene Trevino
2110 Gershwin Ln.
Corpus Christi, TX 78414

Education:
Texas A&M University College Station, Texas 1997 – present
Bachelor of Science degree in ***Biochemistry***
Expected date of graduation: **May 2001**
Cumulative GPA: 3.83

Honors:
Dean's Honor Roll (fall 97 – present)
Howard Hughes Undergraduate Medical Intern (fall 97 – fall 00)
First Place winner at Student Research Week (spring 00)

Professional Experience:
Texas A&M University College Station, Texas 1997-present
Research Assistant with Dr. Nick Pace
Researched hydrogen bonding in proteins as well as increasing the stability of proteins.

Extracurricular Activities:
Aggie Awakening Retreat (spring 98 – summer 99)
Volunteer at St. Joseph Hospital (spring 99 - spring 00)
Member of St. Joseph Catholic Church Folk Choir (spring 98 – present)
Volunteer at St. Mary's Catholic Church Office (fall 97 – spring 98)
Intramural Tennis (summer 99 – present)