# Energetic Analysis of an Engineered Cation−$\pi$ Interaction in Staphylococcal Nuclease

Alice Y. Ting, Injae Shin,† Claudia Lucero, and Peter G. Schultz*

*Howard Hughes Medical Institute, Department of Chemistry University of California, Lawrence Berkeley National Laboratory Berkeley, California 94720*

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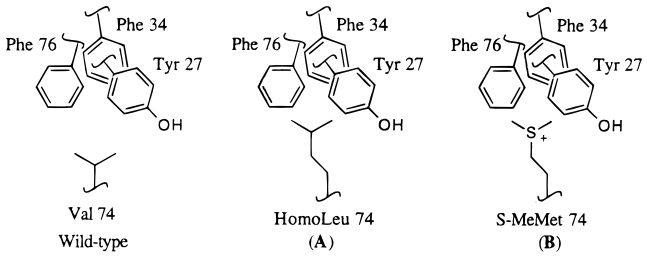
The cation−$\pi$ interaction plays an important role in protein structure, binding, and catalytic function.[1] Despite numerous studies of the energetics of the cation−$\pi$ interaction in synthetic host−guest systems,[2] its energetic value in biological systems is less well understood.[3] The measurement of cation−$\pi$ energies by conventional site-directed mutagenesis is complicated by the absence of naturally occurring positively charged isosteres of neutral amino acids; nonisosteric replacements introduce perturbations that are difficult to quantify or subtract out. Consequently, we have used unnatural amino acid mutagenesis[4] with an isosteric pair of unnatural amino acids[5] to quantify the energetic contribution of an engineered cation−$\pi$ interaction in the interior of staphylococcal nuclease (SNase) to overall protein stability. A hydrophobic pocket composed of two phenylalanine side chains and one tyrosine side chain is occupied in wild-type (WT) SNase by Val$^{74}$ (Scheme 1).[6] This neutral valine residue was replaced with the cationic residue *S*-methylmethionine using suppressor tRNA methodology (Scheme 1, **B**). The neutral isostere of *S*-methylmethionine, homoleucine, was also incorporated into position 74 for purposes of comparison (Scheme 1, **A**). The thermodynamic stabilities of the two mutants (which have near wild-type catalytic parameters) as well as the difference in aqueous solvation energies of the *S*-methylmethionine and homoleucine side chains were measured. An upper limit of 2.6 kcal mol$^{-1}$ was obtained for the energy of the engineered cation−$\pi$ interaction in SNase, in the absence of significant differences in packing interactions for the two mutant proteins.

SNase is a 149 amino acid $Ca^{2+}$-dependent enzyme that hydrolyzes DNA and RNA to give 3′ mono- and dinucleotides.

**Scheme 1.** Schematic Representation of Homoleucine (A) and *S*-Methylmethionine (B) at Position 74 in an Aromatic Cavity of SNase




It is a convenient system in which to examine the cation−$\pi$ interaction because of the cavity formed in its hydrophobic interior by a triad of aromatic residues, Phe$^{34}$, Phe$^{76}$, and Tyr$^{27}$. This cavity is partly occupied by a valine residue whose side chain extends toward the three rings; the distance from side chain to any one ring is 4−5 Å. The aromatic triad, a substructure of the five-strand $\beta$-barrel which forms the major hydrophobic core of SNase, has been studied by alanine replacement mutagenesis, which showed that all three aromatic side chains interact to make an important contribution to overall protein stability.[7]

To engineer a cation−$\pi$ system in SNase, Val$^{74}$ was replaced with a positively charged unnatural amino acid, *S*-methylmethionine (S-MeMet). The trisubstituted sulfonium group of S-MeMet is sterically analogous to the neutral isopropyl functionality of valine;[8] however, to prevent $\beta$-elimination of the sulfonium group, S-MeMet contains a two-carbon linker between the backbone $C_\alpha$ and the sulfonium moiety. Modeling with X-PLOR,[9] indicates that this linker positions the sulfonium group within cation−$\pi$ radius[10] of the aromatic rings. The S-MeMet amino acid was synthesized by S-methylation of *N*-nitroveratryloxycarbonyl-L-methionine cyanomethyl ester with methyl triflate in $CH_2Cl_2$. The sulfonium salt was characterized by $^1$H NMR and mass spectrometry and confirmed to be stable in both hydrophobic and aqueous media at physiological pH.[11] The Val$^{74}$ → S-MeMet mutant (**B**) was synthesized by in vitro transcription/translation of the SNase gene containing an *amber* stop codon at position 74 in the presence of amber suppressor tRNA derived from *Escherichia coli* tRNA$^{Asn}$ that was chemically acylated with S-MeMet.[12] For purposes of comparison, the neutral and isosteric mutant, Val$^{74}$ → homoLeu (**A**), was synthesized in a similar fashion using suppressor tRNA acylated with homoleucine.[13] Both proteins were expressed with C-terminal $His_6$ tags and purified to homogeneity by nickel affinity chromatography.[14] The suppression efficiencies of S-MeMet and homoLeu were 15−20% and 20−25%, respectively. The enzymatic activities of both mutants were measured by the Cuatrecasas spectrophotometric

* To whom correspondence should be addressed.
† Department of Chemistry, Yonsei University, Seoul 120-749, Korea.

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(11) $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 8.21 (1H, d), 7.68 (1H, s), 7.17 (1H, s), 5.38 (2H, q), 5.02 (2H, s), 4.37 (1H, m), 3.94 (3H, s), 3.82 (3H, s), 3.35 (2H, m), 2.87 (6H, s), 2.26 (1H, m), 2.08 (1H, m).
(12) Cload, S. T.; Liu, D. R.; Froland, W. A.; Schultz, P. G. *Chem. Biol.* **1996**, *3*, 1033−8. Optimal in vitro translation conditions for S-MeMet incorporation utilized 11 mM $Mg(OAc)_2$ and 11 mM EGTA.
(13) Optimal in vitro translation conditions for homoLeu incorporation utilized 9 mM $Mg(OAc)_2$ and 12 mM EGTA.
(14) Purity was judged by 15% SDS−PAGE and silver stain.

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**Table 1.** Relative Thermodynamic Stabilities of Proteins Containing Homoleucine and *S*-Methylmethionine and Their Kinetic Constants

| protein | $K_m$[a] | $V_{max}$[b] | $C_m$[c] | $m_{GuHCl}$[d] | $\Delta\Delta G_{H_2O}$[e] |
|---|---|---|---|---|---|
| WT | 107 | 0.81 | 0.69 | 1.00 | 2.11 |
| Val 74 → homoLeu (**A**) | 123 | 0.69 | 0.36 | 1.07 | 0 |
| Val 74 → S-MeMet (**B**) | 139 | 0.48 | 0.41 | 0.69 | −0.73 |

[a] $K_m$ is expressed in units of $\mu$g/mL. The kinetic constants were obtained by measuring the change in absorbance at 260 nm with varying amounts of single-stranded calf thymus DNA (1−50 $\mu$g mL$^{-1}$) in 10 mM $Ca^{2+}$ and 40 mM sodium glycinate, pH 9.9 at 23 °C. [b] $V_{max}$ is expressed in units of $A_{260}$ $\mu g^{-1}$ min$^{-1}$. [c] Midpoint concentration of GuHCl in molarity. [d] Slope of the line fitted to log $K_{app}$ vs [GuHCl] plot. Units are relative to the wild type in vitro expressed protein which is normalized to 1.00. [e] $\Delta\Delta G^0_{H_2O}$(U−F) is expressed in units of kcal mol$^{-1}$. A negative value denotes decreased stability relative to the Val$^{74}$ → homoLeu (**A**) mutant.

assay,[15] and the $K_m$ and $V_{max}$ parameters are reported in Table 1. Because the values are similar to those of WT SNase, the S-MeMet and homoLeu substitutions most likely do not significantly alter the overall structure and function of the enzyme.

The thermodynamic stabilities of WT SNase, the Val$^{74}$ → S-MeMet mutant, and the Val$^{74}$ → homoLeu mutant were determined by measuring the $\Delta G°$(U−F), or free energy difference between unfolded and folded proteins, by the fluorescence quenching method of Shortle and Meeker (Table 1).[16] The $\Delta G°$(U−F) of the homoLeu mutant is smaller than that of WT SNase by 2.11 kcal mol$^{-1}$, probably reflecting the entropic and steric price of replacing Val$^{74}$ in the folded protein with a side chain that is two methylene units longer. The energetic difference between the folded and unfolded S-MeMet mutant, $\Delta G°$(U−F) (Val$^{74}$ → S-MeMet), is 0.73 kcal mol$^{-1}$ less than the corresponding difference for the homoLeu mutant, $\Delta G°$(U−F) (Val$^{74}$ → homoLeu). This value represents the contributions of several factors, including van der Waals interactions, solvation effects, conformational entropy, and electrostatic terms (including the cation−$\pi$ interaction). A number of these factors probably make negligible contributions; for instance, in the unfolded form of SNase, the differences in van der Waals and electrostatic interactions between the Val$^{74}$ → S-MeMet mutant and the Val$^{74}$ → homoLeu mutant are likely to be small if the side chain of residue 74 is solvent-exposed and involved in minimal hydrophobic packing and electrostatic interactions with the rest of the protein. The contribution of conformational entropy to $\Delta\Delta G°$(U−F) is also likely to be negligible because the conformational flexibilities of the side chains of the two mutants are comparable due to their isosteric nature. Thus the measured difference in the stabilities of the Val$^{74}$ → S-MeMet mutant and the Val$^{74}$ → homoLeu mutant mostly reflects the differential cation−$\pi$ and van der Waals interactions in the folded proteins and the differential solvation energies in the unfolded protein forms. The magnitude of the latter term can be approximated by the octanol−water partition coefficients[17] for the side chains of *S*-methylmethionine and homoleucine which are 0.02 and 6.07, respectively.[18] This gives an estimate for the energetic difference between the unfolded forms of the Val$^{74}$ → S-MeMet, Val$^{74}$ → homoLeu mutants of 3.33 kcal mol$^{-1}$ or less, the exact value depending on the degree of water exposure of side chain 74 in the denatured state.

The energy of the engineered cation−$\pi$ interaction can be estimated by subtracting $\Delta\Delta G°$(solvation) (of Ac-[S-MeMet]-OMe relative to Ac-homoLeu-OMe) from $\Delta\Delta G°$(U−F) (of the Val$^{74}$ → S-MeMet mutant relative to the Val$^{74}$ → homoLeu mutant) to obtain a value of 2.6 kcal mol$^{-1}$. This represents an upper limit to the contribution of the engineered cation−$\pi$ interaction to the stability of folded SNase—if the difference in van der Waals forces between the sulfonium mutant and its neutral isostere are minimal in the folded proteins. Both the S-MeMet and homoLeu side chains could be modeled into the hydrophobic core of SNase using X-PLOR without significant perturbations of the aromatic side chains of Phe$^{34}$, Phe$^{76}$, and Tyr$^{27}$. Nonetheless, given the decrease in the stability of the homoLeu mutant relative to WT SNase, and the slightly larger C−S$^+$ vs C−C bond length, it is possible that the $\Delta\Delta G°$(U−F) value of 0.73 kcal mol$^{-1}$ does include a contribution from $\Delta\Delta G°$(F) (van der Waals). If this is the case, the energy of the cation−$\pi$ interaction is greater than 2.6 kcal mol$^{-1}$ by the value of $\Delta\Delta G°$(F) (van der Waals).[19,20]

The value of 2.6 kcal mol$^{-1}$ measured here can be compared to those obtained from other studies of cation−$\pi$ energetics in both synthetic host−guest and biological systems. For example, Dougherty and co-workers measured a minimum value of 2.5 kcal mol$^{-1}$ for the cation−$\pi$ interaction between a synthetic aromatic host and a pyridinium guest,[21] and Schneider and co-workers estimated a cation−$\pi$ binding energy of 0.5 kcal mol$^{-1}$ between a positively charged lipophilic host and each phenyl group of an aromatic guest to which it was complexed.[21] In a study involving the protein barnase, mutagenesis experiments were used to determine that a HisH$^+$-Trp cation−$\pi$ interaction was worth 1.4 kcal mol$^{-1}$ relative to the differential solvation energy in water.[22] The difference between this value and that obtained in the SNase system may reflect differing degrees of solvent accessibility of the cationic amino acids in the folded proteins as well as the nature of the aromatic groups. Thus the cation−$\pi$ interaction can contribute significantly—on the order of a hydrogen bond[23]—to the overall stability of proteins.

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(19) We assume that if there is a difference between the van der Waals interactions in the S-MeMet and homoLeu-containing mutants, then the side chain with the larger van der Waals radius, S-MeMet, destabilizes the folded form of the protein more than the side chain with the smaller radius.

(20) It is also important to note that in the absence of crystallographic data, it is not possible to determine whether the engineered cation−$\pi$ interaction in SNase is mono-, di-, or tridentate, and therefore whether the value of 2.6 kcal mol$^{-1}$ should be divided by two or three to give 1.3 kcal mol$^{-1}$ or 0.87 kcal mol$^{-1}$, respectively, for the energetic value of the cation−$\pi$ interaction.

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