Effects of mutations on the thermodynamics of proteins

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Abstract - Results obtained by means of differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) with several proteins and mutant forms thereof are reported. DSC has been employed primarily to determine the thermodynamic changes accompanying the thermally induced unfolding of proteins, and ITC to measure the thermodynamics of the interactions of proteins with various ligands. It has become increasingly evident that we are dealing with very complicated systems in which a single amino acid replacement probably causes numerous small effects which are widely distributed in the molecule, and which add up to an observed effect that is obviously difficult to rationalize in terms of molecular structure. It is nevertheless important to continue to broaden the base of quantitative information in the field.

INTRODUCTION

Many proteins, nucleic acids and other biopolymers adopt highly specified structures in solution which they retain through various more or less severe perturbations. These structures, which in terms of the free energy changes accompanying their breakdown are only marginally stable, are maintained not only by valence bond forces but also by large numbers of relatively weak forces such as van der Waals interactions, hydrogen bonding and hydrophobic interactions. Our present understanding of the intra- and intermolecular forces at work in biopolymers is largely qualitative in character, and an important problem in biophysics is to give this understanding a more quantitative character.

It would appear that a promising mode of attack on this problem so far as proteins are concerned would be to make known amino acid replacements in a protein and to determine quantitatively the resultant changes in the energetics of processes involving the protein, such as thermal unfolding or the binding of ligands. We and others have attempted to follow this line of attack by making calorimetric measurements on a number of processes involving mutant proteins. In this report I shall summarize measurements we have made employing differential scanning calorimetry and isothermal titration calorimetry. I should perhaps state at the outset that the general problem before us is far more complicated than we had anticipated, and that a great deal more effort will be required before anything approximating a quantitative understanding can be reached.

DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Figure 1 gives a schematic representation of a widely used DSC, the MC-2 manufactured by Microcal, Inc., of Northampton, MA. A solution of a biopolymer fills the sample (right hand) and the solvent, eg. buffer, fills the reference cell. The cells and surrounding adiabatic shield are heated at a constant rate such as 1 K per min. Feedback signals from multijunction thermopiles between the cells, and between the cells and the shield, insure that all parts of the assembly are continually at the same temperature. When an equilibrium process such as protein unfolding or DNA helix-to-coil transition is initiated by the temperature rise, an
absorption of heat will take place in the sample, and the extra power supplied to
the sample cell to maintain equality of temperature between the cells is recorded.
(If heat evolution is observed, it may be concluded that the process under
observation is kinetically rather than thermodynamically limited.)

Figure 2 shows typical DSC curves observed at two different values of the pH for
wild type staphylococcal nuclease (SNase). In each case the solid curve is the
observed data and the dashed curves are the theoretical curve expected for an
assumed model and the calculated baseline. The parameters defining the calculated
curve are evaluated by a least squares curve fitting process (ref. 1). The model
assumed for SNase involves possible dimerization of both the folded (N) and unfolded
(D) forms of the protein:

\[
\frac{K_N}{2} = \frac{K}{N} = \frac{D}{D_2}
\]

It is assumed that \(K_N\) and \(K_D\) are constant over the temperature range of the
unfolding reaction. We take the three parameters controlling the main equilibrium
as \(t_{1/2}\), the temperature (°C) of half-completion of the reaction (Note a), \(\Delta H_{cal}\),
the specific enthalpy (cal g\(^{-1}\)), and \(\beta = \Delta H_{W} / \Delta H_{cal}\). \(\Delta H_{W}\), the van't Hoff enthalpy,
controls the progress of the reaction according to the van't Hoff equation

\[
d \ln K/dT = -\Delta H_{W}/RT^2
\]

For a strictly two-state, all or none, process \(\beta = MW\), the molecular weight, but we
have found it necessary in many cases to allow \(\beta\) to float in the curve fitting
calculation. It is obvious from the curves in Fig. 2 that there are substantial
permanent specific heat increases, \(\Delta C_p\), due to the transitions, which of course must
be included in the curve fitting process.

We have run DSC experiments on numerous wild type (WT) and mutant proteins, in order
to measure the effects of mutations on the changes in standard free energy,
enthalpy, standard entropy and heat capacity due to protein unfolding. A convenient

\[\text{Note a: In this paper } t \text{ refers to temperatures in } °C \text{ and } T = t + 273.15; \text{ enthalpies}\]
\[\text{are expressed in calories, with } 1 \text{ cal = 4.184 joules.}\]
way to express the effect of a mutation is in terms of $\Delta t_{1/2} = t_{1/2}^{\text{mutant}} - t_{1/2}^{\text{WT}}$ and $\Delta A_d = A_d^{\text{mutant}} - A_d^{\text{WT}}$, where $A_d$ is $\Delta G_d$, $\Delta H_d$, $\Delta S_d$ or $\Delta C_p$, calculated to $t_{1/2}^{\text{WT}}$.

**EFFECTS OF MUTATIONS IN T4 LYSOZYME**

We have received copious amounts of the lysozyme of phage T4 and 17 mutant forms from B. Matthews and J. Wozniak of the University of Oregon. All of these proteins showed reversible transitions which could be fit within a standard deviation of 1-2% of the maximal value of the excess specific heat by theoretical curves based on a modified two-state model with the parameter $\beta$ allowed to float. The results obtained at pH 2.5 and 46.2°C ($t_{1/2}$ for the WT protein at pH 2.5) are summarized in Table 1. Values of $\Delta G_d$ ranging from -4.0 to +0.85 kcal mol$^{-1}$ were observed; a negative value indicates destabilization of the folded form, stabilization of the unfolded form, or a mixture of these two.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\Delta G_d$ (kcal mol$^{-1}$)</th>
<th>$\Delta H_d$ (kcal mol$^{-1}$)</th>
<th>$\Delta C_p$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R96H</td>
<td>-12.2</td>
<td>-4.0</td>
<td>6</td>
</tr>
<tr>
<td>I3P</td>
<td>-9.2</td>
<td>-3.0</td>
<td>3</td>
</tr>
<tr>
<td>I3T</td>
<td>-6.4</td>
<td>-2.5</td>
<td>11</td>
</tr>
<tr>
<td>T157L</td>
<td>-5.9</td>
<td>-1.7</td>
<td>-9</td>
</tr>
<tr>
<td>T157I</td>
<td>-5.8</td>
<td>-1.9</td>
<td>1</td>
</tr>
<tr>
<td>T157V</td>
<td>-4.9</td>
<td>-1.6</td>
<td>-1</td>
</tr>
<tr>
<td>T157E</td>
<td>-4.7</td>
<td>-1.8</td>
<td>-12</td>
</tr>
<tr>
<td>T157N</td>
<td>-4.4</td>
<td>-1.3</td>
<td>-12</td>
</tr>
<tr>
<td>T157F</td>
<td>-4.3</td>
<td>-1.5</td>
<td>-14</td>
</tr>
<tr>
<td>C54T</td>
<td>-2.8</td>
<td>-0.9</td>
<td>3</td>
</tr>
<tr>
<td>C79A</td>
<td>-2.3</td>
<td>-0.8</td>
<td>6</td>
</tr>
<tr>
<td>T157R</td>
<td>-1.7</td>
<td>-0.6</td>
<td>2</td>
</tr>
<tr>
<td>A82P</td>
<td>1.4</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>A93P</td>
<td>1.4</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>G113A</td>
<td>1.5</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>I3L</td>
<td>2.8</td>
<td>0.85</td>
<td>9</td>
</tr>
</tbody>
</table>

**Table 2. Changes in thermodynamic parameters for protein unfolding at pH 7.0, 51.4°C, caused by various mutations in staphylcoccal nuclease.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\Delta G_d$ (kcal mol$^{-1}$)</th>
<th>$\Delta H_d$ (kcal mol$^{-1}$)</th>
<th>$\Delta C_p$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L25A</td>
<td>-11.5</td>
<td>-2.6</td>
<td>11</td>
</tr>
<tr>
<td>A90S</td>
<td>-10.7</td>
<td>-2.5</td>
<td>13</td>
</tr>
<tr>
<td>G79S</td>
<td>-7.6</td>
<td>-1.3</td>
<td>-14</td>
</tr>
<tr>
<td>V66L</td>
<td>3.9</td>
<td>0.8</td>
<td>14</td>
</tr>
<tr>
<td>G88V</td>
<td>4.7</td>
<td>0.3</td>
<td>-52</td>
</tr>
<tr>
<td>G88V</td>
<td>2.9</td>
<td>0.5</td>
<td>-17</td>
</tr>
<tr>
<td>V66L</td>
<td>3.9</td>
<td>0.5</td>
<td>-14</td>
</tr>
<tr>
<td>G88V</td>
<td>6.4</td>
<td>1.0</td>
<td>-24</td>
</tr>
<tr>
<td>H124L</td>
<td>5.1</td>
<td>1.3</td>
<td>2</td>
</tr>
</tbody>
</table>

*SNase with residues 44-49 deleted (ref. 7)

Estimated uncertainties as for Table 1.

Estimated uncertainties: $\Delta t_{1/2}$, ±0.2°C; $\Delta G_d$, ±0.4 kcal mol$^{-1}$; $\Delta H_d$, ±4 kcal mol$^{-1}$; $\Delta C_p$, ±200 cal K$^{-1}$mol$^{-1}$.

Attempts based on theoretical approaches have been made to rationalize these values for $\Delta G_d$ (not values for $\Delta H_d$) in terms of the three dimensional structures of these mutants. For example, Tidor and Karplus (ref. 2) undertook a simulation-protein dynamics analysis of the R96H replacement. The complexity of such an analysis is illustrated by the fact that 44 contributions to the free energy change on converting Arg to His in the folded protein and 30 to the conversion in the unfolded form were included, giving an overall free energy change in fair agreement with our experimental value. In this analysis only interactions involving residues 93 through 99 were included. No indication of the enthalpy and entropy make-up of these free energy values was included.

Weaver and Matthews (ref. 3) determined by x-ray crystallography the structure of R96H to a resolution of 1.7Å. These authors concluded on the basis of comparisons with the structure of the WT protein, that the two most important sources of the apparent destabilization of the mutant were the loss of a helix-dipole interaction involving the C-terminus of helix 82-90, and significant strain caused by the introduction of the imidazole ring of histidine. Tidor and Karplus, on the other hand, state that the helix-dipole model is inappropriate for this case.
It should be emphasized here that the changes in free energy, which are the quantities usually considered in connection with the effects of mutations on the thermodynamics of protein unfolding, show little or no correlation with the effects on the enthalpy or entropy of unfolding. This important point is illustrated in Fig. 3 in which $\Delta \Delta G^0$ and $\Delta \Delta H$ at pH 2.5 are plotted for 12 mutants of T4 lysozyme. The values for $\Delta \Delta G^0$ are uniformly quite small while those for $\Delta \Delta H$ are usually much larger and in eight cases of opposite sign. In the Thr 157 series, for example, it is interesting that T157I and T157L have nearly equal values of $\Delta \Delta G^0$ but values of $\Delta \Delta H$ differing by 10 kcal mol$^{-1}$ and 32 cal K$^{-1}$ mol$^{-1}$ respectively. The x-ray data of Alber et al. (ref. 4) show that the introduction of Ile at position 157 forces the side chain of Asp159 to move 1.1Å from its position in the WT protein, a much larger motion than caused by any of the other T157 substitutions. It is accordingly difficult to understand why the substitution of Leu at position 157 causes a much larger change in enthalpy than the introduction of either Ile or Val.

**EFFECTS OF MUTATIONS IN STAPHYLOCOCCAL NUCLEASE**

We have studied by means of DSC (ref. 5) several mutants of staphylococcal nuclease (SNase), namely L25A, V66L, G79S, G88V, A90S and H124L, the multiple mutants V66L:G88V, and V66L:G79S:G88V (ref. 6), and an unusual mutant, ASNase (ref. 7), formed by removing residues 44-49 that form a loop in the WT protein. It was found with all of these proteins that $t_1/2$ decreases significantly as the concentration is increased, indicating that the unfolded form of the protein is more aggregated than the folded form. In fact, good curve fits were obtained in most cases on the assumption that the folded form is monomeric while the unfolded form is nearly completely dimerized. We have arbitrarily selected 500 μM as a standard concentration at which quantities such as $\Delta \Delta G^0$ are calculated and compared.

The changes in thermodynamic quantities resulting from the various mutations are listed in Table 2. The first point which may be made here is that the data for the double and triple mutations indicate approximate additivity of $\Delta \Delta G^0$ and $\Delta \Delta H$. For the double mutant the calculated and observed values for $\Delta \Delta G^0$ are respectively 1.3 and 1.0, and for $\Delta \Delta H$ -31 and -24 (all in kcal mol$^{-1}$); similarly for the triple mutant the figures are 0 and 0.3 and -45 and -52. Approximate additivity in the effects of mutations has been observed in other cases. As was the case with the mutants of T4 lysozyme, there is no discernible correlation between the values for $\Delta \Delta G^0$ and those for $\Delta \Delta H$, as illustrated in Fig. 4. An extreme example of this is shown by the triple mutant for which the entropy of unfolding at pH 7.0 is reduced from 228 to 68 cal K$^{-1}$ mol$^{-1}$! What might appear to be a very minor disturbance of the energetics of the molecule, as judged by the value of $\Delta \Delta G^0 = 0.3$ kcal mol$^{-1}$, obviously involves profound changes in enthalpy and entropy which are probably distributed throughout both the folded and unfolded forms of the molecule. There is evidence, for example, based on CD observations, small angle x-ray scattering and NMR (J. Flanagan, unpublished results) that the β-barrel portion of the molecular structure is actually tightened by the triple mutation, and this could lead to a decrease in configurational entropy of the unfolded triple mutant compared to the unfolded WT protein.
Effects of mutations on the thermodynamics of proteins

In collaboration with R. Sauer and his colleagues at M.I.T. we have looked at 14 single replacements and 1 double replacement in the N-terminal domain of the λ repressor of E.coli (refs. 8, 9, and 10). The results observed with 6 of these mutants are shown in Fig. 5 and the complete results are summarized in Table 3. It was found with several of the proteins that after scanning to 60°C and then cooling to 20°C for 2 or 3 hours, the N-terminal unfolding peak was reproduced on rescanning. It is evident in the figure that the unfolded N-terminal domain has no significant effect on the unfolding of the C-terminal domain, as would be expected on the basis of Brandts' model for domain interaction (ref. 11). The one apparent exception, A66T, was found after the DSC result became available to have a previously undetected mutation, Y210H, in the C-terminal domain. The mutations causing the largest destabilizations are at buried sites. All the N-terminal peaks, except for A66T, and all the C-terminal peaks are hypersharp and unsymmetrical for unknown reasons; only the A66T peak satisfies the two-state criterion of \( \Delta H_{VH} = \Delta H_{\text{cal}} \).

Table 3 lists the data for 8 mutants causing apparent stabilization and 7 destabilizing mutants. Two of the latter cause unusually large drops in \( t_{1/2} \). It is interesting that replacing His by Phe in the second of these causes a weak stabilization.

Replacement of Gly 46 by Ala in the third α-helix in the N-terminal domain led to an apparent stabilization of 0.7 kcal mol\(^{-1}\), and Gly 48 by Ala, Asn or Ser to stabilizations of 0.9, 0.8, or 0.7 kcal mol\(^{-1}\), respectively (ref. 10). The double mutant with both of these Gly's replaced by Ala, showed stabilization of 1.1 kcal
mol\(^{-1}\), with rough additivity. These results are consistent with the usual view (ref. 12) that the Gly residue is a poor helix former while the Ala residue is one of the best, and the Asn and Ser residues are intermediate.

Table 3. Changes in the thermodynamic parameters for protein unfolding at pH 8.0, 53.4\(^\circ\)C, caused by various mutations in the N-terminal domain of \(\lambda\) repressor.

<table>
<thead>
<tr>
<th>Protein</th>
<th>(\Delta t_{1/2}^\circ) ((^\circ)C)</th>
<th>(\Delta G_2^\circ) (kcal mol(^{-1}))</th>
<th>(\Delta H_4^\circ) (kcal mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y22H</td>
<td>-22.7</td>
<td>-2.2</td>
<td>-36</td>
</tr>
<tr>
<td>A66T</td>
<td>-22.5</td>
<td>-2.8</td>
<td>-26</td>
</tr>
<tr>
<td>I84S</td>
<td>-14.3</td>
<td>-2.2</td>
<td>-17</td>
</tr>
<tr>
<td>A49V</td>
<td>-13.0</td>
<td>-1.2</td>
<td>-37</td>
</tr>
<tr>
<td>E83K</td>
<td>3.6</td>
<td>-0.7</td>
<td>7</td>
</tr>
<tr>
<td>E34K</td>
<td>-1.9</td>
<td>-0.3</td>
<td>-15</td>
</tr>
<tr>
<td>Q44Y</td>
<td>-0.1</td>
<td>-0.1</td>
<td>6</td>
</tr>
<tr>
<td>Y22F</td>
<td>1.9</td>
<td>0.3</td>
<td>-10</td>
</tr>
<tr>
<td>K4Q</td>
<td>2.0</td>
<td>0.4</td>
<td>4</td>
</tr>
<tr>
<td>G46A</td>
<td>3.1</td>
<td>0.7</td>
<td>4</td>
</tr>
<tr>
<td>G48S</td>
<td>4.0</td>
<td>0.7</td>
<td>-11</td>
</tr>
<tr>
<td>G48N</td>
<td>4.1</td>
<td>0.8</td>
<td>-4</td>
</tr>
<tr>
<td>G48A</td>
<td>4.7</td>
<td>0.9</td>
<td>-4</td>
</tr>
<tr>
<td>Q33Y</td>
<td>5.9</td>
<td>1.3</td>
<td>8</td>
</tr>
<tr>
<td>G46A:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G48A:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G48A:</td>
<td>6.2</td>
<td>1.1</td>
<td>-7</td>
</tr>
</tbody>
</table>

Table 4. Changes in thermodynamic parameters for the binding of modified S-peptide to S-protein at 25\(^\circ\)C produced by amino acid replacements in 16-residue S-peptide.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>(\Delta G^\circ) (kcal mol(^{-1}))</th>
<th>(\Delta H^\circ) (kcal mol(^{-1}))</th>
<th>(T\Delta S^\circ) (cal K(^{-1}) mol(^{-1}))</th>
<th>(\Delta C_P^\circ) (cal K(^{-1}) mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13A</td>
<td>4.0</td>
<td>5.0</td>
<td>-10</td>
<td>50</td>
</tr>
<tr>
<td>M13F</td>
<td>2.7</td>
<td>4.4</td>
<td>-1.7</td>
<td>20</td>
</tr>
<tr>
<td>M13ANB*</td>
<td>1.3</td>
<td>9.5</td>
<td>8.2</td>
<td>200</td>
</tr>
<tr>
<td>M13NOR**</td>
<td>3.6</td>
<td>4.0</td>
<td>3.6</td>
<td>-150</td>
</tr>
<tr>
<td>M138</td>
<td>-0.3</td>
<td>5.8</td>
<td>5.7</td>
<td>100</td>
</tr>
<tr>
<td>M13I</td>
<td>3.3</td>
<td>5.8</td>
<td>6.1</td>
<td>250</td>
</tr>
<tr>
<td>M13V</td>
<td>-2.4</td>
<td>4.8</td>
<td>5.2</td>
<td>270</td>
</tr>
</tbody>
</table>

\(*\alpha\)-Amino-n-butyric acid
\(**\)Norleucine

Estimated uncertainties as for Table 1.

**PREDICTION OF MUTATIONAL EFFECTS**

In the preceding sections relatively little attention has been given to attempts to rationalize the observed mutational effects, largely because, in the opinion of the present author, there is not available at present an adequate quantitative understanding of the intramolecular forces at work. However, there are many cases where, at least in qualitative terms, predicted changes in stability have been realized. For example, Stearman et al. (ref. 13), working with the separated dimeric N-terminal domain of \(\lambda\)-repressor, introduced the G46A and G48A mutations mentioned above, which in the holoprotein produced a total increase of 6.2\(^\circ\)C in \(t_{1/2}\) (ref. 10), and in addition a Y88C mutation which permitted formation of an intersubunit disulfide bond. It had previously been shown (ref. 14) that this mutation caused an increase in \(t_{1/2}\) at 8\(^\circ\)C. The protein containing all three mutations showed \(\Delta t_{1/2}^\circ = 16\)^\circ\)C, a very substantial apparent stabilization, probably including a contribution from decreased configurational entropy of the unfolded state.

The ability to predict mutations which should lead to significant increases in the thermal stability of proteins might well be of considerable pharmacological or other importance. However, in this connection it should not be overlooked that nature has already designed some very stable proteins. An outstanding example of this is the tryptophan aporepressor of \(E.\) coli, hardly a thermophilic bacterium (ref. 15). This protein is a dimer of molecular weight 24.7 kDa, containing no metal or other non-amino acid component, and of ordinary appearing amino acid sequence. Yet when it is heated at pH 7.5 in 10 mM phosphate buffer containing 100 mM KCl, it undergoes unfolding and dissociation into monomeric units in a transition centered at 93\(^\circ\)C. If the heating is interrupted at 100\(^\circ\)C and the solution cooled to 50\(^\circ\)C during 20 min, the unfolding-dissociation transition is found to be quantitatively repeated on reheating. It is also important to keep in mind that so far as thermal stability is concerned attacks other than that of introducing mutations can have profound effects. For example Santoro et al. (ref. 16) found that N-methylglycine (sarcosine) at a concentration of 8.2 M raises the denaturational temperature of ribonuclease A by 22\(^\circ\)C, remarkably with no change in the enthalpy of the reversible unfolding, and very little change in enzymic activity.
Effects of mutations on the thermodynamics of proteins

ISOThERMAL TITRATION CALORIMETRY

ITC is a very useful method for the investigation of the thermodynamics of reactions, having as its main advantage over other isothermal methods that enthalpies are directly determined. Titration calorimeters have been developed to the point that a total enthalpy change of a few hundred microcalories is adequate for obtaining 20 or more points on a titration curve in the period of 60-90 min provided the reaction being studied has a half time of less than a minute.

A schematic diagram of a widely used titration calorimeter, the Omega, manufactured by Microcal of Northampton, MA, is shown in Figure 6 (ref. 19). The cell on the right is filled with substrate solution (about 1.3mL) and the one on the left with reference liquid (water if aqueous titrations are being run). Titrant is added in 3 to 10 μL injections from a microsyringe actuated by a computer-controlled stepping motor. Electrical feedback insures rapid return to the baseline after each injection. A typical experimental record obtained with this instrument is reproduced in Figure 7, the upper panel showing the peaks produced by the injections of titrant and the lower panel the integrated heat of each injection and the titration curve fitted to these heats by a least squares procedure. From a titration such as this, one obtains the binding constant, the standard free energy of binding and the enthalpy of binding. The heat capacity change and the van't Hoff enthalpy can be evaluated by running titrations over a temperature range.

THE BINDING OF MODIFIED S-PEPTIDES TO S-PROTEINS TO FORM RIBONUCLEASE-S

In 1959, Richards and Vithayathil (ref. 20) observed that mild proteolysis of RNase A severed one peptide bond between residues 20 and 21. The product, RNase S, had full enzymatic activity and unchanged x-ray structure. At low pH the peptide and the protein can be separated, and at neutral pH they recombine to form RNase S' which is indistinguishable from RNase S. We have used the Omega calorimeter to study in detail the binding of modified S-peptides to S-protein to form modified RNase S'. In our work (refs. 21 and 22) we have used abbreviated peptides lacking the residues 16 to 20, which do not appear to have much effect on the binding, with Met13 replaced by various other residues.
The binding constants found for this system at pH 7.0 and 25°C ranged from $7.7 \times 10^3 \text{M}^{-1}$ for M13A to $9.2 \times 10^6 \text{M}^{-1}$ for M13V. Unusually large enthalpy changes are seen, ranging from -39 kcal mol$^{-1}$ for M13 to -30 kcal mol$^{-1}$ for M13ANB (a-amino-n-butyric acid). A major source of these enthalpy changes, as shown by DSC curves for the S-protein and RNase S', is the refolding of the S-protein induced by the binding of the S-peptide. Unusually large decreases in entropy were also observed, as required by the Second Law. Table 4 lists the changes in thermodynamic parameters caused by the various replacements of M13 in the S-peptide. It is evident that, as seen for the thermal unfolding of mutant proteins, there is no correlation between the values for $\Delta G^\circ$ and $\Delta H^\circ$. Actually, in this system the values for $\Delta G^\circ$ cover a much wider fractional change, -0.3 to 4.0 kcal mol$^{-1}$, than do the values for $\Delta H^\circ$. It will probably be difficult to account quantitatively for a 4.3 kcal mol$^{-1}$ difference between the free energies for M13 and M13A. It seems likely that the changes, and the differences in the changes, in RNase S' caused by the binding of the various peptides are widely distributed in the protein molecule.

Acknowledgements
The people from whom we have received generous supplies of proteins have been mentioned above. Discussions with Drs. D. Engelman and F. Richards of Yale University and Dr. J. Brandts of the University of Massachusetts have been very useful. Most of the actual experimentation has been carried out by my colleagues Drs. S.-J. Bae, P. Connelly, J. Planagan, L. Ghosaini, C.-Q. Hu, N. Kishore, S. Kitamura, J. Ladbury, A. Tanaka, J. Thomson, and R. Varadarajan. Our research is supported by the National Institutes of Health, the National Science Foundation, and Yale University.

REFERENCES