The thermodynamic mechanism of protein stabilization by trehalose

Guifu Xie*, Serge N. Timasheff

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254, USA

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Abstract

The stabilization of ribonuclease A by α-α-trehalose was studied by preferential interaction and thermal unfolding. The protein is stabilized by trehalose at pH 2.8 and pH 5.5. Wyman linkage analysis showed increased exclusion of trehalose from the protein domain on denaturation. Preferential interaction measurements were carried out at 52°C at pH 5.5 and 2.8, where the protein is native and unfolded, respectively, and at 20°C where the protein is native at both pH values. At the low temperature, the interaction was preferential exclusion. At 52°C the interaction was that of preferential binding, greater to the native than the unfolded protein, the increment on denaturation being identical to that deduced from the Wyman analysis. The stabilizing effect of trehalose can be fully accounted by the change in transfer free energy on unfolding. The temperature dependence of the preferential interactions of 0.5 M trehalose with ribonuclease A showed that it is the smaller preferential binding to the unfolded protein than to the native one which gives rise to the stabilization. A thermodynamic analysis of the data led to approximate values of the transfer enthalpies and transfer entropies for the trehalose–ribonuclease A system.

Keywords: Trehalose; Preferential interactions; Transfer free energy; Transfer enthalpy; Transfer entropy; Protein stabilization; Co-solvents

1. Introduction

It is well known that the native conformation of many proteins and enzymes can be stabilized by polyhydric molecules such as glycerol and sucrose at high concentration (≥ 1 M) [1–15]; and Xie and Timasheff, manuscript in preparation. Studies on the mechanism of interaction of these compounds with proteins have shown that, at room temperature, they are preferentially excluded from contact with the protein surface [10,12–15]. This means that the addition of these co-solvents to an aqueous protein solution is thermodynamically unfavorable since it raises the chemical potential of both the protein and the additive.

The sugar, α, α-trehalose, α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside, is a carbohydrate found in living organisms. It is a nonreducing glucose disaccharide commonly found at high concentrations in anhydrobiotic organisms (as high as 20% of the dry weight) [16–19]. It has been identified as being of potential biotechnological importance, due to its effectiveness in stabilizing membrane structure in the dry state, inhibiting biological damage at low temperature and stabilizing protein structure during freezing and freeze drying [19–22]. The detailed
mechanism by which trehalose produces these stabilizing effects has not yet been established. It is known that trehalose binds to the head group of lipids in dry bilayers [23], but the nature of the molecular interactions in solution is not yet known [24–26]. Trehalose has a greater ability to preserve membranes in anhydrobiotic organisms and to stabilize enzymes during the freeze-drying procedure than glycerol, glucose, sucrose and galactose [19,20,22].

Thermodynamic analyses of the interactions of sucrose, glucose and lactose [10,12] have led to the conclusion that these sugars stabilize the native structure of proteins by inducing a greater preferential hydration of denatured, unfolded proteins than of the native structure. Yet this hypothesis has not been rigorously tested. It seemed of interest, therefore, to examine the detailed mechanism of this stabilization by carrying out parallel preferential interaction measurements of the protein with sugar solutions in both the native and denatured states. Trehalose was selected due to its chemically inert nature. The results of these studies are presented in this paper.

2. Materials and methods

2.1. Materials

\(\alpha,\alpha\)-Trehalose dihydrate was purchased from Pfaniastei Laboratories Inc. The pure product is a white fine powder with an absorbance at 277 nm of a 0.5 M aqueous solution lower than 0.04. Some lots were rejected, since the absorbance value was as high as 0.10 and the solution was yellow. Because of its high cost, the trehalose was recycled after use by recrystallizing it three times from distilled water. Ribonuclease A (RNase A) was purchased from Sigma (type II A from bovine pancreas). This protein was further purified on a Sulfoethyl-Sephadex c-25 column in 0.1 M, pH 6.47 phosphate buffer (Xie and Timasheff, manuscript in preparation), following essentially the procedure of Crestfield et al. [27,28]. Glycine was the reagent grade product from Sigma. The molecular-porous membrane tubing (23 \times 100, M.W. cutoff: 6,000–8,000) was purchased from Spectrum. Fisher standardized 1 N HCl solution was used for the pH adjustment of solutions. All solutions contained 0.04 M glycine at pH 2.8, or 0.04 M sodium acetate at pH 5.5. The protein concentrations were determined by UV absorbance using an extinction coefficient of RNase A of 7.06 dL g\(^{-1}\) cm\(^{-1}\) at pH 2.8 and 7.12 dL g\(^{-1}\) cm\(^{-1}\) at pH 5.5 at 277 nm. The absorbance values of the protein in concentrated trehalose can be expected to be somewhat different from those in dilute buffer. For 0.9 M trehalose, the protein absorbance values were 7.10 dL g\(^{-1}\) cm\(^{-1}\) and 7.20 dL g\(^{-1}\) cm\(^{-1}\) for pH 2.8 and pH 5.5, respectively. While this may affect slightly the absolute values of the partial specific volumes, it should have no effect on the preferential interaction parameters.

2.2. Thermodynamics of thermal denaturation

The change in protein absorbance with changing temperature was followed on a Gilford Response II UV/Vis spectrophotometer at 287 nm. In all experiments, 1 mg ml\(^{-1}\) RNase was used. The solutions were degased for 3–5 min by using a water pump to prevent the production of bubbles on heating. The temperature change steps were programmed in such a way that the rate of heating was \(\sim 0.35^\circ\)C min\(^{-1}\). This had the consequence that in the full heating–cooling cycles the protein solutions spent up to 4 h at high temperature (> 40°C), since heating was continued up to 85°C before being reversed. In all cases, the cooling curves were mirror images of the heating curves. Since the interaction measurements required the protein solutions to be kept at 52°C for 20 h, control experiments were carried out following this regime. The transition curves obtained after cooling for 2 h were normal, which indicated that no irreversible changes had taken place.

2.3. Preferential interactions

The preferential interactions of the solvent components with the protein were obtained from the partial specific volumes of the protein measured with a Precision DMA-02 density meter (Anton Paar, Gratz). All measurements were made at 20°C following the procedures published previously [12,29–31]. Buffer solutions containing a given trehalose concen-
tration were filtered through a sintered-glass filter. Each preferential interaction point requires that the apparent partial specific volume be measured with and without dialysis against the solvent at the experimental temperature, giving, in turn, values at constant chemical potential, $\phi_2$, and constant molality, $\phi_2^c$, of co-solvent. To this end, for each point, parallel experiments were carried out. In one set, the protein solutions were dialyzed for 20 h at the experimental temperature, say 52°C. In the other set, the protein solutions were kept for 20 h at the same temperature without dialysis. Since the density measurements, which are a co-solvent concentration measurement, were carried out at 20°C, both solutions were then cooled to 20°C for 2–3 h, the one that had been dialyzed at high temperature having been transferred out of the dialysis bag. Specifically, for each point in constant molality experiments, six samples (6–20 mg) of RNase were dried in small glass test tubes over phosphorus pentoxide at 40°C for 2 days in a vacuum oven. After cooling to room temperature, about 1 ml of solution was added, sealed tightly immediately with Parafilm and left to stand overnight at the experimental temperature. When the samples had to be kept in a water bath, the tubes were sealed by 5 layers of Parafilm and 2 layers of Saran Wrap. Following this, the samples were kept at 20°C for 3–4 h prior to the densimetry experiments. In constant chemical potential experiments, i.e., at dialysis equilibrium with the co-solvent, each of seven samples of protein (8–25 mg) was dissolved in about 1.1 ml solvent solution and transferred into a dialysis bag, the molecular-porous membrane tubing, which had been boiled and rinsed in deionized water. The samples were dialyzed at the desired temperature for about 20 h against 800–1000 ml of co-solvent solution and then kept for 3–4 h at 20°C in small tubes that had been sealed tightly to avoid evaporation. Following the density measurement, each protein solution was diluted gravimetrically with solvent to a final optical density of about 0.7 (~1 mg ml$^{-1}$). The concentrations were determined spectrophotometrically with a Perkin Elmer Lambda 3B UV/Vis spectrophotometer. Individual $\phi_2^o$ and $\phi_2^{\infty}$ points were measured in up to four independent experiments. The results were found to be highly reproducible both at low and high temperature.

2.4. Calculation of preferential interaction parameters

The partial specific volume of a protein is obtained from density measurements with the equation [32]

$$\phi = 1/\rho_o \times [1 - (\rho - \rho_o)/c]$$ (1)

where $\phi$ is the apparent specific volume, $\rho$ and $\rho_o$ are the densities of the protein solution and reference solvent, respectively, and $c$ is the protein concentration in g ml$^{-1}$. $\phi_2^o$ and $\phi_2^{\infty}$ were determined under conditions at which the molalities of the solvent components and their chemical potentials were, in turn, kept identical in the protein solution and in the reference solvent. Using the notation of Scatchard [33] and Stockmayer [34], component 1 = water, component 2 = protein, component 3 = additive (here trehalose), the preferential binding parameter, $(\partial g_3/\partial g_2)_{T, \mu_i, \mu_3}$, was calculated by [35]

$$(\partial g_3/\partial g_2)_{T, \mu_i, \mu_3} = \rho_o (\phi_2^o - \phi_2^{\infty})/(1 - \rho_o \bar{\nu}_3)$$ (2)

where $g_i$ is the concentration of component $i$ in grams per gram of water, $T$ is the thermodynamic (Kelvin) temperature, $\mu_i$ is the chemical potential of component $i$, $\bar{\nu}_3$ is the partial specific volume of component 3. The superscript o indicates extrapolation to zero protein concentration $^1$. The values of $\rho_o$ and $\bar{\nu}_3$ used are given in Table 1. The preferential binding parameter, $(\partial g_3/\partial g_2)_{T, \mu_2, \mu_3}$, expresses the excess of component 3 in the immediate domain of the protein with reference to its concentration in the bulk solvent. A positive value of this parameter means an excess of component 3, while a negative

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$^1$The measured values of $\phi_2$ and $\phi_2^c$ were found to be independent of protein concentration, whether the protein solution was heated or not. This is a strong indication that any effects observed are not the consequence of protein self-association or aggregation, which, by the Law of Mass Action, must increase with protein concentration. For a reversible self-association, its effects would be, furthermore, eliminated by extrapolation to zero protein concentration. Random, non-specific irreversible aggregation (coagulation) has, in previous experience, led to erratic, poorly reproducible values of the apparent partial specific volumes. Furthermore heating at 52°C did not generate any measurable turbidity at 450 nm.
Table 1
Characteristic parameters of trehalose solutions

<table>
<thead>
<tr>
<th>Trehalose/M</th>
<th>g₅/g</th>
<th>m₅/(mol/1000g H₂O)</th>
<th>ρ₅/mL g⁻¹</th>
<th>ε₅/μl g⁻¹</th>
<th>γ₅</th>
<th>(∂lnγ₅/∂m₅)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.9998 ± 0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.09/14</td>
<td>0.269</td>
<td>1.02/12 ± 0.0002</td>
<td>0.600</td>
<td>1.018</td>
<td>0.11/1</td>
</tr>
<tr>
<td>0.3</td>
<td>0.1095</td>
<td>0.320</td>
<td>1.0407 ± 0.0002</td>
<td>0.662</td>
<td>1.058</td>
<td>0.177</td>
</tr>
<tr>
<td>0.4</td>
<td>0.1493</td>
<td>0.436</td>
<td>1.0542 ± 0.0001</td>
<td>0.603</td>
<td>1.080</td>
<td>0.177</td>
</tr>
<tr>
<td>0.5</td>
<td>0.1909</td>
<td>0.558</td>
<td>1.0676 ± 0.0001</td>
<td>0.604</td>
<td>1.104</td>
<td>0.177</td>
</tr>
<tr>
<td>0.7</td>
<td>0.2804</td>
<td>0.819</td>
<td>1.0942 ± 0.0001</td>
<td>0.606</td>
<td>1.156</td>
<td>0.177</td>
</tr>
</tbody>
</table>

value indicates its deficiency in the domain of the protein, i.e., an excess of component 1, water. The corresponding preferential hydration parameter, Email: (\frac{\partial g_1}{\partial g_2})_{T,\mu_1,\mu_2} = -(\frac{\partial g_3}{\partial g_2})_{T,\mu_1,\mu_2} (3)

In molal units, preferential binding is given by

\begin{equation}
(\frac{\partial m_3}{\partial m_2})_{T,\mu_1,\mu_3} = -(\frac{\partial g_3}{\partial g_2})_{T,\mu_1,\mu_2} (4)
\end{equation}

where \( m_i \) is the molality of component \( i \) and \( M_i \) is its molecular weight. The preferential binding parameter is a direct expression of the mutual perturbation of the chemical potentials of components 2 and 3 [38-40]

\begin{equation}
(\frac{\partial g_1}{\partial g_2})_{T,\mu_1,\mu_2} = -(\frac{\partial g_3}{\partial g_2})_{T,\mu_1,\mu_2} (5)
\end{equation}

\begin{equation}
\times [nRT/m_3 \ln(1 - \alpha) - nRT(\frac{\partial \ln g_3}{\partial m_3})_{T,\mu_1,\mu_2}] (6)
\end{equation}

where \( R \) is the universal gas constant, \( n = 1 \) for trehalose, and \( Y_i \) is the activity coefficient of component \( i \). The values listed in Table 1 were calculated from osmotic coefficient data of Lin and Timasheff (to be published) using the relation

\begin{equation}
\ln Y_3 = (\phi_3 - 1) + \int_0^{m_3} [\frac{(\phi_3 - 1)}{m_3}] dm_3 (7)
\end{equation}

where \( \phi_3 \) is the osmotic coefficient of the trehalose solution. The value of

\begin{equation}
(\frac{\partial \ln Y_3}{\partial m_3})_{T,\mu,\mu_2} (8)
\end{equation}

was found to be constant at 0.177 for the entire trehalose concentration range used in this study.

3. Results

3.1. Thermal stabilization

The thermal transition curves, both heating and cooling, of RNase A in varying amounts of trehalose at pH 2.8 (A and B) and pH 5.5 (C and D) are shown in Fig. 1. It is clear that addition of trehalose causes an elevation of the thermal transition temperature of RNase A. Taking the midpoint of the transition as the transition temperature (\( T_m \)), it can be seen that the thermal transition temperature increased monotonically with trehalose concentration, as shown in Table 2, the increase being greater at pH 2.8 than at pH 5.5: at 0.9 M trehalose, the transition temperature was raised by 7.8°C at pH 2.8, but only by 5.5°C at pH 5.5. The curves of Fig. 1 were analyzed in terms of a two-state transition, \( N = \alpha \). The relative amounts of the protein in the native (\( \alpha \)) and unfolded states (1 - \( \alpha \)) were estimated from the UV absorbance, following the procedure of Biltonen and Lumry [41], and the equilibrium constant

\[ K = \alpha/(1 - \alpha) \]

was calculated at each trehalose concentration as a function of temperature. From these values, the enthalpy of unfolding as a function of solvent composition was obtained from fitting to a truncated form of the integrated Van't Hoff equation [12,42-44]

\[ \ln K = a + b(1/T) + c\ln T \]

\[ \Delta H^\circ = R(cT - b) \]

\[ \Delta C_p = Rc \]
Fig. 1. Thermal transitions of RNase A. (A), pH 2.8, 0.04 M glycine buffer, heating. (B), same as (A), cooling. (C), pH 5.5, 0.04 M sodium acetate buffer, heating. (D), same as (C), cooling. The trehalose concentrations are listed on the figure.

Table 2
Thermodynamic parameters of thermal denaturation of RNase A at pH 2.8 and pH 5.5 in trehalose–water systems

<table>
<thead>
<tr>
<th>Trehalose/M</th>
<th>( T_m )/°C Heating (unfolding)</th>
<th>( T_m )/°C Cooling (refolding)</th>
<th>( \Delta H^\circ ) at ( T_m )/kcal mol(^{-1})</th>
<th>( \Delta G^\circ )/20°C/kcal mol(^{-1})</th>
<th>( \delta(\Delta G^\circ)/(20°C)/kcal mol(^{-1})</th>
<th>( \delta(\Delta G^\circ)/(52°C)/kcal mol(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>40.9 ± 0.3</td>
<td>40.5 ± 0.4</td>
<td>82.7 ± 5</td>
<td>5.51 ± 0.3</td>
<td>0 ± 0.3</td>
<td>0 ± 0.3</td>
</tr>
<tr>
<td>0.1</td>
<td>41.8</td>
<td>41.6</td>
<td>82.8</td>
<td>5.72</td>
<td>0.21</td>
<td>0.23</td>
</tr>
<tr>
<td>0.2</td>
<td>42.2</td>
<td>42.0</td>
<td>83.6</td>
<td>5.92</td>
<td>0.41</td>
<td>0.37</td>
</tr>
<tr>
<td>0.3</td>
<td>43.4</td>
<td>43.1</td>
<td>83.9</td>
<td>6.19</td>
<td>0.68</td>
<td>0.62</td>
</tr>
<tr>
<td>0.4</td>
<td>44.1</td>
<td>43.8</td>
<td>84.5</td>
<td>6.43</td>
<td>0.92</td>
<td>0.83</td>
</tr>
<tr>
<td>0.5</td>
<td>45.0</td>
<td>45.0</td>
<td>85.3</td>
<td>6.69</td>
<td>1.18</td>
<td>1.03</td>
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<td>0.6</td>
<td>45.9</td>
<td>45.9</td>
<td>86.8</td>
<td>7.04</td>
<td>1.53</td>
<td>1.25</td>
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<td>0.7</td>
<td>47.0</td>
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<td>1.87</td>
<td>1.40</td>
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<td>0.8</td>
<td>47.7</td>
<td>47.6</td>
<td>89.8</td>
<td>7.75</td>
<td>2.24</td>
<td>1.72</td>
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<tr>
<td>0.9</td>
<td>48.7</td>
<td>48.5</td>
<td>90.4</td>
<td>8.06</td>
<td>2.55</td>
<td>2.00</td>
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<td>pH 5.5</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.0</td>
<td>60.7 ± 0.3</td>
<td>59.4 ± 0.4</td>
<td>108.3 ± 4</td>
<td>13.15 ± 0.4</td>
<td>0 ± 0.4</td>
<td>0 ± 0.4</td>
</tr>
<tr>
<td>0.1</td>
<td>61.1</td>
<td>60.1</td>
<td>107.0</td>
<td>13.20</td>
<td>0.05</td>
<td>0.07</td>
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<tr>
<td>0.2</td>
<td>61.7</td>
<td>61.1</td>
<td>108.4</td>
<td>13.47</td>
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<td>0.34</td>
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<tr>
<td>0.3</td>
<td>62.3</td>
<td>61.8</td>
<td>107.7</td>
<td>13.50</td>
<td>0.35</td>
<td>0.44</td>
</tr>
<tr>
<td>0.4</td>
<td>62.9</td>
<td>62.1</td>
<td>106.0</td>
<td>13.71</td>
<td>0.56</td>
<td>0.67</td>
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<tr>
<td>0.5</td>
<td>63.3</td>
<td>62.6</td>
<td>105.6</td>
<td>13.78</td>
<td>0.67</td>
<td>0.73</td>
</tr>
<tr>
<td>0.6</td>
<td>64.0</td>
<td>62.9</td>
<td>104.7</td>
<td>13.86</td>
<td>0.71</td>
<td>0.92</td>
</tr>
<tr>
<td>0.7</td>
<td>64.8</td>
<td>64.2</td>
<td>107.0</td>
<td>14.14</td>
<td>0.99</td>
<td>1.25</td>
</tr>
<tr>
<td>0.8</td>
<td>65.4</td>
<td>64.8</td>
<td>106.9</td>
<td>14.30</td>
<td>1.15</td>
<td>1.42</td>
</tr>
<tr>
<td>0.9</td>
<td>66.2</td>
<td>65.8</td>
<td>110.4</td>
<td>14.99</td>
<td>1.84</td>
<td>1.81</td>
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\( ^a \) The values of \( \Delta G^\circ \) in water at 52°C were -2.91 at pH 2.8 and 2.81 at pH 5.5.
where $\Delta H^\circ$ is the standard enthalpy change for thermal unfolding. The values of $\Delta H^\circ$ for RNase A appear to have little dependence on trehalose concentration both at pH 2.8 (A) and pH 5.5 (B), as shown in Table 2. There was no detectable curvature in the van’t Hoff plots. This is similar to the results obtained for RNase A in sorbitol solutions at pH 2.0 and pH 5.5 (Xie and Timasheff, manuscript in preparation). The resulting thermodynamic parameters are summarized in Table 2. The stabilizing effect of trehalose was expressed by $\delta(\Delta G^\circ) = \Delta G^\circ$ (sorbitol solution) $- \Delta G^\circ$ (dilute buffer). It is seen that the values calculated at two temperatures at both pH 2.8 and 5.5 show a consistently significant stabilization that increases with an increase in trehalose concentration.

3.2. Preferential interactions between trehalose and RNase A in the native and denatured states

The contribution of the interaction of solvent components with a protein to its stabilization can be established by preferential binding measurements to the protein in the two states at identical temperature [45]. This, by necessity, requires that the two studies be carried out at different values of pH. The thermal transition data of Fig. 1 and Table 2 permitted the identification of such conditions as 52°C where RNase A is native at pH 5.5 and unfolded at pH 2.8. To ascertain the suitability of these conditions, it was necessary to establish first that the preferential interactions with the native protein are identical at the two pH values. This was done by carrying out dialysis equilibrium experiments with the native protein at 20°C at pH 2.8 and 5.5. The results are presented in Tables 3 and 4 and Fig. 2A. It is quite clear that, at room temperature, the preferential binding values, $(\delta g_3/\delta g_2)_{T,S,W}$, are essentially identical at pH 5.5 and 2.8. With the assumption that this identity of preferential interactions at room temperature is also valid at 52°C, it was possible to compare directly the measurements at the high temperature at the two pH values as those of the two states of the protein. This was further supported by the temperature study described later in which the preferential interactions remained identical up to the onset of the unfolding at pH 2.8.

Examination of Tables 3 and 4 shows that at 20°C
Table 4  
Preferential interaction parameters of RNase A with aqueous trehalose solutions at pH 5.5

<table>
<thead>
<tr>
<th>Trehalose/M</th>
<th>$\phi_2$ (g/g)</th>
<th>$\phi_3$ (g/g)</th>
<th>$\frac{\partial g_1}{\partial g_2}$</th>
<th>$\frac{\partial m_3}{\partial m_2}$</th>
<th>$\frac{\partial m_3}{\partial m_2}$</th>
<th>$\frac{\partial m_3}{\partial m_2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C</td>
<td></td>
<td></td>
<td>(g/mol)</td>
<td>(mol/mol)</td>
<td>(mol/mol)</td>
<td>(mol/mol)</td>
</tr>
<tr>
<td>0.2</td>
<td>0.699 ± 0.001</td>
<td>0.712 ± 0.001</td>
<td>-0.0348 ± 0.0027</td>
<td>+0.487 ± 0.037</td>
<td>-1.39 ± 0.11</td>
<td>+371 ± 29</td>
</tr>
<tr>
<td>0.3</td>
<td>0.698 ± 0.001</td>
<td>0.720 ± 0.001</td>
<td>-0.0613 ± 0.0028</td>
<td>+0.560 ± 0.025</td>
<td>-2.45 ± 0.11</td>
<td>+426 ± 19</td>
</tr>
<tr>
<td>0.4</td>
<td>0.698 ± 0.001</td>
<td>0.724 ± 0.002</td>
<td>-0.0752 ± 0.0045</td>
<td>+0.504 ± 0.029</td>
<td>-3.01 ± 0.11</td>
<td>+383 ± 22</td>
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<td>0.5</td>
<td>0.698 ± 0.002</td>
<td>0.735 ± 0.001</td>
<td>-0.1112 ± 0.0030</td>
<td>+0.583 ± 0.016</td>
<td>-4.45 ± 0.12</td>
<td>+443 ± 12</td>
</tr>
<tr>
<td>0.7</td>
<td>0.698 ± 0.002</td>
<td>0.744 ± 0.001</td>
<td>-0.1364 ± 0.0049</td>
<td>+0.486 ± 0.017</td>
<td>-4.56 ± 0.20</td>
<td>+370 ± 13</td>
</tr>
<tr>
<td>52°C</td>
<td></td>
<td></td>
<td>(g/mol)</td>
<td>(mol/mol)</td>
<td>(mol/mol)</td>
<td>(mol/mol)</td>
</tr>
<tr>
<td>0.2</td>
<td>0.700 ± 0.002</td>
<td>0.696 ± 0.004</td>
<td>+0.0054 ± 0.0080</td>
<td>-0.075 ± 0.112</td>
<td>+0.21 ± 0.31</td>
<td>-57 ± 85</td>
</tr>
<tr>
<td>0.3</td>
<td>0.700 ± 0.004</td>
<td>0.695 ± 0.003</td>
<td>+0.0139 ± 0.0098</td>
<td>-0.127 ± 0.089</td>
<td>+0.56 ± 0.39</td>
<td>-97 ± 67</td>
</tr>
<tr>
<td>0.4</td>
<td>0.699 ± 0.003</td>
<td>0.684 ± 0.005</td>
<td>+0.0434 ± 0.0116</td>
<td>-0.291 ± 0.078</td>
<td>+1.74 ± 0.47</td>
<td>-221 ± 59</td>
</tr>
<tr>
<td>0.5</td>
<td>0.699 ± 0.003</td>
<td>0.674 ± 0.009</td>
<td>+0.0752 ± 0.0180</td>
<td>-0.394 ± 0.094</td>
<td>+3.01 ± 0.72</td>
<td>-300 ± 72</td>
</tr>
</tbody>
</table>

a cal (mol of trehalose)$^{-1}$ (mol of protein)$^{-1}$ in 1000 g H2O.
b The values of $\frac{\partial m_3}{\partial m_2}$ at 20°C: 2890, 1923, 1439, 1147 and 814 cal (mol of trehalose)$^{-1}$ in 1000 g H2O for 0.2, 0.3, 0.4, 0.5 and 0.7 M trehalose solutions, respectively. And the values of $\frac{\partial m_3}{\partial m_2}$ at 52°C: 3206, 2133, 1596 and 1272 cal (mol of trehalose)$^{-1}$ in 1000 g H2O for 0.2, 0.3, 0.4 and 0.5 M trehalose solutions, respectively.

the preferential interactions assume progressively negative values with an increase in trehalose concentration, the values at the two pH values remaining essentially identical. Thus, at 0.2 M trehalose, $\frac{\partial m_3}{\partial m_2}$ is $-1.4$ moles sugar per mole protein and decreases to $-5.46$ moles sugar per mole protein at 0.7 M trehalose. At 52°C, however, the interaction is one of preferential binding at all
trehalose concentrations. The binding is stronger to
the native protein than to the unfolded form. At 0.5
M trehalose, it has values of 3.0 moles of sugar per
mole protein at pH 5.5 and 1.56 moles sugar per
mole protein at pH 2.8. Comparison of the interac-
tions in terms of preferential hydration, shown in
Fig. 2B, demonstrates strikingly that the protein
maintains a level of preferential hydration which is
identical at pH 2.8 and 5.5 and does not vary signifi-
cantly with trehalose concentration. The value of
the preferential hydration (0.49–0.58 grams water per
gram protein) is similar to values determined by Bull
and Breese [46] and Kuntz [47] for a number of
proteins. Its constancy indicates essentially total ex-
clusion of trehalose from contact with the native
protein. Quantitatively, this can be expressed by the
relation between preferential binding and site occu-
pancy by water and co-solvent [37,48–50]
\[
\left( \frac{\partial g_3/\partial g_2}{T_{\rho,\mu,\nu}} \right) = A_3 - g_3 A_1
\]
where \( A_j \) is the amount of component \( i \) that is
bound to (occupies sites on) component 2 as a time
average, expressed in grams of \( i \) per gram of com-
ponent 2. Thermodynamically, \( A_1 \) and \( A_3 \) are related by the exchange equilibria implicit in
\[
\left( \frac{\partial g_3/\partial g_2}{T_{\rho,\mu,\nu}} \right) = \begin{cases} 3.13 + 5.57m_3 \\ 3.13m_3, + 2.79m_3^2 - 1.44m_3^3 \end{cases}
\]
Native protein (pH 2.8, 20°C):
\[
\Delta \mu = 3.13m_3, + 2.79m_3^2 - 1.44m_3^3
\]
Native protein (pH 5.5, 20°C):
\[
\Delta \mu = 3.80 + 1.30m_3
\]
(12)
Native protein (pH 5.5, 22°C):
\[
\Delta \mu = 0.53 - 3.78m_3
\]
(13)
Denatured protein (pH 2.8, 52°C):
\[
\Delta \mu = 1.02 - 3.76m_3
\]
(14)
Denatured protein (pH 2.8, 52°C):
\[
\Delta \mu = 1.02m_3 - 1.88m_3^2 - 1.06m_3^3
\]
(15)
(16)
Therefore, a minor shift in the
relative affinities of water or trehalose for some loci
on the protein surface (by ~ 0.05 kcal mol\(^{-1}\)) can
lead to a shift in the measured preferential interac-
tions including a change in sign.

3.3. Transfer free energy

The free energy of interaction between protein
and co-solvent, with water as reference state, is the
transfer free energy, \( \Delta \mu = \mu_2 - \mu_1 \) (solvent of a given composition) – \( \mu_2 \) (water).
This is obtained by integration of the preferential
interaction parameter, \( \left( \frac{\partial \mu_3}{\partial m_3} \right)_{T,\rho,\mu,\nu} \), which is re-
lated by Eq. (5) to the binding measured by dialysis equilibri-

\[
\Delta \mu = \int_0^{m_3} \left( \frac{\partial \mu_3}{\partial m_3} \right)_{T,\rho,\mu,\nu} dm_3
\]
(10)
The values of the preferential interaction parameter, \( \left( \frac{\partial \mu_3}{\partial m_3} \right)_{T,\rho,\mu,\nu} \), presented in Fig. 3, were fitted
to quadratic forms in trehalose concentration and
integrated according to Eq. (10). The resulting data-
fitting equations are listed:
Native protein (pH 2.8, 20°C):
\[
\Delta \mu = 3.13m_3, + 2.79m_3^2 - 1.44m_3^3
\]
The transfer free energies of RNase A from water to the sugar solution, $\Delta \mu_2 = \mu_{2,s} - \mu_{2,w}$, are presented in Fig. 4 as a function of trehalose concentration. It is evident that at 20°C the interactions of the native protein with trehalose solutions at both pH values are thermodynamically unfavorable relative to pure water, as $\Delta \mu_2$ assumes progressively increasing positive values with increasing sugar concentration. At the high temperature, however, 52°C, the situation is reversed. For the native protein (pH 5.5), $\Delta \mu_2$ is essentially zero up to 0.3 M trehalose, which means that the protein is thermodynamically indifferent to contact with water or sugar solution. Above this solvent composition, the transfer free energy becomes negative, and attains a value of $-5.1$ kcal mol$^{-1}$ at 0.9 M, i.e., contact between the protein and aqueous trehalose becomes highly favorable. The unfolded protein, however, exhibits a less favorable pattern of interactions with the sugar solutions. In fact, after assuming a pattern of weak repulsion, $\Delta \mu_2 = 0.1$ kcal mol$^{-1}$ at 0.3 M trehalose, the interaction becomes increasingly favorable above 0.45 M, but attains a value of only $-2.6$ kcal mol$^{-1}$ at 0.9 M. It is this less favorable interaction of trehalose with denatured than with native protein which causes it to stabilize proteins against unfolding, when water is taken as the reference state.

3.4. Temperature dependence of preferential interactions

The measurement of the transfer free energies of the protein in the native and denatured states into a
cosolvent system of a given composition gives the stabilizing ability of the given co-solvent. A full understanding of the thermodynamic mechanism also requires knowledge of the corresponding enthalpic and entropic parameters. This requires that the preferential interactions be measured as a function of temperature. Such measurements were, therefore, performed on the interactions of RNase A with 0.5 M trehalose at pH 2.8 and 5.5. The thermal transition data of Fig. 1 show that at pH 5.5, the protein was in the native state at temperatures below 54°C (the transition takes place between 54.0°C and 72.4°C). However, at pH 2.8, denaturation set in above 35°C (the transition region is between 34.2°C and 54.8°C). Therefore, dialysis equilibrium experiments were carried out on RNase A in 0.5 M trehalose between 4.0°C and 58.0°C at both pH values. At the high pH this gave values of the preferential interactions with native protein as a function of temperature. At pH 2.8, however, the preferential interaction measurements scanned over the transition of the protein, native below 34°C, and denatured above 52°C, after passing through the zone of transition.

The results of these measurements, presented in Table 5, show that at isomolal conditions, the apparent partial specific volume remains essentially invariant with temperature at pH 5.5. At pH 2.8, it also was constant below the zone of transition, but increased slightly as the protein unfolded. At isopotential conditions, this parameter, $\phi_2^P$, changed drastically with temperature, as its value decreased sharply. As shown in Fig. 5A, the decrease in $\phi_2^P$ was stronger at pH 5.5 than at pH 2.8. At low temperature, the values of $\phi_2^P$ were identical at the two pH values up to 30°C. Above this temperature, $\phi_2^P$ of the native protein started to decline sharply. At the

<table>
<thead>
<tr>
<th>Temperature/°C</th>
<th>$\phi_2^P$/g (ml of trehalose)-1 (mol of protein)-1 in 1000 g H$_2$O.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2.8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.700 ± 0.001 0.737 ± 0.002 0.583 ± 0.024 4.45 ± 0.18 4824 ± 196</td>
</tr>
<tr>
<td>15</td>
<td>0.700 ± 0.001 0.736 ± 0.003 0.567 ± 0.031 4.33 ± 0.24 4880 ± 271</td>
</tr>
<tr>
<td>20</td>
<td>0.700 ± 0.002 0.736 ± 0.003 0.567 ± 0.039 4.33 ± 0.30 4967 ± 345</td>
</tr>
<tr>
<td>25</td>
<td>0.701 ± 0.001 0.736 ± 0.006 0.551 ± 0.031 4.21 ± 0.24 4913 ± 281</td>
</tr>
<tr>
<td>30</td>
<td>0.702 ± 0.002 0.735 ± 0.009 0.520 ± 0.055 3.97 ± 0.42 4719 ± 509</td>
</tr>
<tr>
<td>35</td>
<td>0.702 ± 0.002 0.734 ± 0.010 0.504 ± 0.079 3.85 ± 0.60 4793 ± 749</td>
</tr>
<tr>
<td>40</td>
<td>0.701 ± 0.001 0.733 ± 0.010 0.488 ± 0.097 3.72 ± 0.82 4867 ± 987</td>
</tr>
<tr>
<td>45</td>
<td>0.700 ± 0.001 0.732 ± 0.012 0.477 ± 0.119 3.60 ± 1.04 4943 ± 124</td>
</tr>
<tr>
<td>50</td>
<td>0.700 ± 0.001 0.732 ± 0.014 0.465 ± 0.143 3.48 ± 1.25 5019 ± 147</td>
</tr>
<tr>
<td>55</td>
<td>0.700 ± 0.001 0.732 ± 0.017 0.453 ± 0.168 3.37 ± 1.47 5094 ± 168</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 5.5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.698 ± 0.001 0.735 ± 0.002 0.583 ± 0.024 4.45 ± 0.18 4824 ± 196</td>
</tr>
<tr>
<td>15</td>
<td>0.698 ± 0.001 0.735 ± 0.003 0.567 ± 0.031 4.33 ± 0.24 4880 ± 271</td>
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<tr>
<td>20</td>
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</tr>
<tr>
<td>25</td>
<td>0.697 ± 0.002 0.735 ± 0.008 0.551 ± 0.031 4.21 ± 0.24 4913 ± 281</td>
</tr>
<tr>
<td>30</td>
<td>0.697 ± 0.002 0.732 ± 0.011 0.520 ± 0.055 3.97 ± 0.42 4719 ± 509</td>
</tr>
<tr>
<td>35</td>
<td>0.697 ± 0.002 0.731 ± 0.015 0.504 ± 0.079 3.85 ± 0.60 4793 ± 749</td>
</tr>
<tr>
<td>40</td>
<td>0.697 ± 0.002 0.731 ± 0.017 0.488 ± 0.097 3.72 ± 0.82 4867 ± 987</td>
</tr>
<tr>
<td>45</td>
<td>0.697 ± 0.002 0.731 ± 0.019 0.477 ± 0.119 3.60 ± 1.04 4943 ± 124</td>
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<tr>
<td>50</td>
<td>0.697 ± 0.002 0.731 ± 0.021 0.465 ± 0.143 3.48 ± 1.25 5019 ± 147</td>
</tr>
<tr>
<td>55</td>
<td>0.697 ± 0.002 0.731 ± 0.024 0.453 ± 0.168 3.37 ± 1.47 5094 ± 168</td>
</tr>
</tbody>
</table>

The values of $\phi_2^P$ were 1084, 1127, 1147, 1167, 1188, 1206, 1214, 1237, 1245, 1255, 1258, 1272 and 1296 cal (mol of trehalose)-2 in 1000 g H$_2$O for 0.5 trehalose at 4, 15, 20, 25, 30.5, 35, 37, 43, 45, 47.5, 48.5, 52 and 58°C, respectively.
acid pH, this decline set in only above 45°C, i.e. after the onset of the unfolding transition.

The preferential interaction parameters deduced from these measurements are presented in Table 5. An identical pattern of a decrease in preferential exclusion and preferential hydration with rising temperature is quite evident. The preferential trehalose binding, \((\partial g_3/\partial g_2)_{T,\mu_1,\mu_2}\), is compared for the two pH values in Fig. 5B. The values are negative and identical at the two pH values below 35°C, where they are essentially constant. Above this temperature they diverge as the values measured at pH 5.5 start increasing and become positive at temperatures above 45°C. The same is true at pH 2.8, but only above

Fig. 5. Temperature dependence of: (A) the apparent partial specific volume of RNase A at dialysis equilibrium; (B) the preferential binding of co-solvent to RNase A in 0.5 M trehalose solution. (○) pH 5.5; (●) pH 2.8.

Fig. 6. Temperature dependence of: (A) the preferential hydration of RNase A in 0.5 M trehalose solution; (B) the total binding (site occupancy) of co-solvent to RNase A in 0.5 M trehalose solution. (○) pH 5.5; (●) pH 2.8.
52°C. This means that the interaction of the co-solvent with the protein is greater with the native protein than with the denatured form. Expressed in terms of preferential hydration, shown in Fig. 6A, these results indicate that hydration of the protein remains constant below 35°C. Analysis in terms of Eq. (9) gives the results of Fig. 6B. These show that below 35°C there is total exclusion of trehalose from the immediate domain of the protein. Using a value of 0.49 grams water per gram protein for \( A_1 \), which is the average measured at 20°C (Tables 3 and 4), results in a slightly negative value of the total binding of trehalose to RNase A. Above 35°C, however, there sets in a sharp increase for the native protein which at 52°C attains a value of \( A_3 \) of 0.17 grams trehalose per gram protein for the native protein. \( \Delta \) 6.8 molecules of trehalose penetrate the hydration layer and enter into contact with the protein even though the hydration remains at a constant high value. For the denatured protein, pH 2.8, at 52°C \( A_1 \) attains a value of only 0.13 grams trehalose per gram protein, or 5.3 molecules of trehalose that make contact with one molecule of protein.

### 3.5. Thermodynamic parameters of the preferential interactions

The transfer free energy, \( \Delta \mu_2 \), which is the change in the partial molal free energy, \( \bar{C}_2 \), of the protein when transferred from water to the co-solvent system, is the sum of the contributions of the partial molal enthalpy, \( \bar{H}_2 \), and the partial molal entropy, \( \bar{S}_2 \). Therefore, knowledge of the concentration dependence of the preferential interaction parameter as a function temperature should permit the deduction of the necessary enthalpy and entropy parameters, since

\[
(\partial \mu_2 / \partial m_3)_{T,P,m_2} = (\partial \bar{H}_2 / \partial m_3)_{T,P,m_2} - T(\partial \bar{S}_2 / \partial m_3)_{T,P,m_2} \quad (15)
\]

The temperature dependence of \((\partial \mu_2 / \partial m_3)_{T,P,m_2}\), at the two pH values is given in Fig. 7. Its analysis in terms of Eq. (8), in which \( \ln K \) is replaced by

\[
-\frac{1}{RT} \left( \frac{\partial \mu_2}{\partial m_3} \right)
\]

yields \((\partial \bar{H}_2 / \partial m_3)_{T,P,m_2}\) and \((\partial \bar{S}_2 / \partial m_3)_{T,P,m_2}\). The results of the calculations for native RNase A (pH 5.5) are shown in Fig. 8. Panel A gives the Van't Hoff plot of the preferential interaction parameter, while Fig. 8B gives the temperature dependence of the variation of the partial molal enthalpy with trehalose concentration obtained by applying Eq. (8) to the data of Fig. 8A. This parameter is found to increase with temperature, with values of \(-87\) kcal mol\(^{-1}\) at 4°C and 236 kcal mol\(^{-1}\) at 58°C, which gives a strongly positive value of the variation with co-solvent concentration of the partial molal heat capacity, 6.0 kcal deg\(^{-1}\) (mol prot\(^{-1}\) (mol co-solvent\(^{-1}\)). It is interesting to note that these values are similar to those reported for the closely related osmolyte sorbitol. At 25°C, the value of \((\partial \bar{H}_2 / \partial m_3)_{T,P,m_2}\) for the interaction of 0.5 M trehalose with RNase A is 30 kcal mol\(^{-2}\). For 30% sorbitol, it is 28 kcal mol\(^{-2}\) when the protein is RNase A (Xie and Timasheff, to be published), and 48 kcal mol\(^{-2}\) when the protein is bovine serum albumin [53a].

The transfer enthalpy, \( \Delta \bar{H}_{2,m_2} \), and transfer entropy, \( \Delta \bar{S}_{2,m_2} \), can be calculated by integrating \((\partial \bar{H}_2 / \partial m_3)_{T,P,m_2} \) over \( dm_3 \), since

\[
\Delta \bar{H}_{2,m_2} = \Delta \bar{H}_{2,m_1} = \bar{H}_{2,m_1} - \bar{H}_{2,w} = \int_0^{m_3} \frac{\partial \bar{H}_2}{\partial m_3} dm_3 \quad (16a)
\]

\[
T\Delta \bar{S}_{2,m_2} = \Delta \bar{H}_{2,m_1} - \Delta \mu_{2,ir} \quad (16b)
\]

Fig. 7. Temperature dependence of the preferential interaction parameter, \((\partial \mu_2 / \partial m_3)_{T,P,m_2}\), in 0.5 M trehalose solution, (O), pH 5.5; (●), pH 2.8. The dotted line is the parameter calculated at 52°C for the denatured protein (see text).
Fig. 8. Thermodynamics of the preferential interactions of aqueous trehalose solutions with native RNase A. (A) Van't Hoff plot of the transfer free energy variation with trehalose concentration: (▼) 0.2 M trehalose; (▼) 0.3 M trehalose; (□) 0.4 M trehalose; (●) 0.5 M trehalose. (B) Temperature dependence of the variation of the transfer enthalpy with trehalose concentration at 0.2 M (▼), 0.3 M (▼), 0.4 M (□), 0.5 M (●) trehalose. The numbers on the figure are the slopes, which represent (δμ2/δm3)x,p,α, in cal deg⁻¹ (mol prot⁻¹) (mol co-solvent)⁻¹. C. Dependence on trehalose concentration of the transfer enthalpy variation with trehalose concentration at 20°C and 52°C. The dotted line is the parameter calculated at 52°C for the denatured protein. D. Dependence of the transfer enthalpy on trehalose concentration at 20°C and 52°C. The dotted line is the parameter calculated at 52°C for the denatured protein. E. Temperature dependence of the variation of the transfer entropy with trehalose concentration at 0.5 M trehalose. F. Dependence of the transfer entropy on trehalose concentration at 20°C and 52°C.

In the absence of detailed measurements of the temperature dependence of preferential interactions at several co-solvent concentrations, an approximate calculation was carried out with the assumption that the form of the temperature dependence of (δμ2/δm3) is the same at all co-solvent concentrations. To that end the data at 20°C and 52°C at 0.2, 0.3 and 0.4 M trehalose were plotted in a manner similar to Fig. 8A and subjected to the Van't Hoff analysis of Eq. (8). The resulting values are shown in
Fig. 8B as a function of temperature and in Fig. 8C as a function of trehalose concentration. Integration according to Eq. (16a) gave the values \( \Delta H_c \) presented in Fig. 8D. Application of Eq. (16b) to the data of Fig. 7 and Fig. 8B, as well as those of Fig. 8D and Fig. 4 gave the entropy parameters of Fig. 8E and F. While the numbers shown in Fig. 8C, D, and F are highly approximate and should be regarded as indicative of trends, nevertheless they are useful in permitting some qualitative conclusions. With this caution, it can be stated that the transfer of RNase A from water into aqueous trehalose at high temperature is accompanied by large concentration-dependent positive changes in enthalpy and entropy which are close to compensating, while at room temperatures these changes are much smaller, reflecting the positive value of \( \Delta C_{p,2} \).

4. Discussion

In the absence of some non-identified and non-detected, highly reproducible artifact or secondary effect, which mimics exactly the linkage between protein unfolding and changes in preferential interactions, the current results give a full, even though in parts highly approximate, description of the thermodynamics of the stabilization of a protein (RNase A) by an osmolyte (trehalose) in thermal unfolding. Let us examine this in terms of the linkage concept taking as reference state first the solvent of a given composition and then dilute aqueous buffer, i.e. water.

4.1. Wyman analysis of the stabilization

The effect of a ligand on any equilibrium at a given concentration of ligand, which is the reference state, is expressed by the Wyman linkage equation \([45, 53-56]\]

\[
\frac{\partial \ln K}{\partial \ln a_3} = \left( \frac{\partial m_3}{\partial m_2} \right)_{T, P, \mu}^D - \left( \frac{\partial m_3}{\partial m_2} \right)_{T, P, \mu}^N
\]

(17)

where \( a_3 \) is the activity of component 3, the cosolvent, and D and N refer specifically to the denaturation equilibrium N \( \rightleftharpoons \) D. Therefore, the slope of a log-log plot of the equilibrium constant versus the activity of the co-solvent, trehalose in the present case, gives the change in preferential binding of the ligand to the protein that accompanies the transformation of the protein. A plot of the data of Fig. 1 in terms of Eq. (17) is given in Fig. 9. Values of slopes are written on the figure. The negative values show that denaturation of RNase A in the presence of trehalose is accompanied by a decrease in the preferential binding of trehalose to the protein, and that this effect increases with trehalose concentration. Specifically, at 52°C, for 0.5 M trehalose, the slope has a value of \(-1.40 \pm 0.05\) moles trehalose per mole protein. This should be equal to the difference between the preferential bindings measured directly on the two end states of the reaction. The data of Tables 3 and 4 give values of \( (\partial m_3/\partial m_2)_{T, P, \mu} \) of 3.01 moles trehalose per mole protein for the native protein (pH 5.5) and 1.56 moles of trehalose per
mole of protein for the unfolded form (pH 2.8). Their difference, $1.56 - 3.01 = -1.45$ moles trehalose per mole protein, is essentially identical with that given by the Wyman slope. This identity shows that stabilization of RNase A by trehalose is modulated by general weak protein–co-solvent interactions, expressed through preferential binding. The small value of the change in preferential interactions in fact reflects the change in the actual number of trehalose–protein contacts which have much greater numerical values. Coming back to the decomposition of the preferential interactions into total protein surface occupancy by water and trehalose, as expressed through Eq. (9), gives \( A_3 - A_3^N = 5.32 - 6.76 = -1.44 \) molecules of trehalose occupying sites on a protein molecule. The much larger values of \( A_3 \) than \( \langle \rho m_3 / \rho m_2 \rangle_{T,n,m} \) reflect the fact that the latter is a pure thermodynamic number that encompasses water–trehalose exchange at loci on the protein surface, as well as non-exchangeable water [45,51–53]. In addition to \( A_3 \), \( \langle \rho m_3 / \rho m_2 \rangle_{T,n,m,\Lambda_3} \) contains some 450 molecules of water that occupy sites on the protein surface. On the other hand, site-occupancy values are non-thermodynamic quantities that reflect protein–ligand contacts that may be detected by some particular techniques, such as calorimetry or spectroscopy, but which, by neglecting exchange with water, do not provide a measure of the global interactions of the protein surface with its environment [45,53]. Finally, the results of the present study clearly demonstrate that stabilization of the native protein structure does not require preferential exclusion of the co-solvent from the protein surface. In the present example, at 52°C trehalose is preferentially bound to both the native and denatured proteins. What provides the stabilization is the difference between these two quantities. Preferential binding to the denatured protein is smaller than that to the native form. Hence, there is stabilization. At lower temperatures, trehalose is preferentially excluded from native protein. For example, at 43°C, the preferential interaction with native protein (pH 5.5) is $-1.80$ moles of sugar per mole of protein. Wyman analysis gives a slope of $-1.72$ moles sugar per mole of protein. This predicts that the preferential interaction with denatured protein would be one of exclusion at a level of $-3.5$ moles of sugar per mole of protein. The finding that at low temperature stabilization is determined by an increase in preferential exclusion of trehalose on denaturation of RNase A, while at 52°C it is determined by a decrease in preferential binding of trehalose to the protein, does not imply in any way a change in the mechanism of stabilization. These observations are a trivial consequence of the fact that preferential interaction, exclusion or binding, is simply a measure of the gradient with co-solvent concentration of the transfer free energy (see Eq. (5) and Eq. (10)). Preferential interaction indicates, therefore, whether \( \Delta \mu_2 \) is increasing or decreasing at the given co-solvent concentration. Stabilization with respect to water, which is the commonly sought parameter, is given by the difference between the transfer free energies of the denatured and native proteins. (For a detailed discussion, see Refs. [45,53]).

4.2. Stabilization relative to pure aqueous medium

Integration of the Wyman equation (Eq. (11)) shows that, with water as the reference state, stabilization expressed as the increment of the standard free energy of denaturation in a given medium over that in pure water, \( \delta \Delta G^o = \Delta G^o_w - \Delta G^o_w \), is equal to the increment in transfer free energy upon denaturation, \( \delta \Delta \mu_2 = \Delta \mu_2^D - \Delta \mu_2^N \), i.e.

\[
\delta \Delta G^o = \Delta G^o_w - \Delta G^o_w = \Delta \mu_2^D - \Delta \mu_2^N = \delta \Delta \mu_2
\]

The thermal unfolding results given in Fig. 1 and Table 2 were combined with the transfer free energies calculated for native and denatured protein at 52°C from the preferential interaction data of Tables 3 and 4 with the use of Eq. (13) and Eq. (14). The results are summarized in Table 6. It is seen that, at all trehalose concentrations, the two sides of Eq. (18), after rearrangement into a thermodynamic box, are identical within experimental error. Therefore, the change in transfer free energy is sufficient to account for the stabilization. This means that there is no need to invoke any specific interactions, such as binding of the sugar at particular structure stabilizing loci, to account for its action. General, non-specific thermodynamic interaction is sufficient.
The stabilization, expressed by the change in transfer free energy upon denaturation, is a resultant of such changes in the transfer enthalpy and transfer entropy during denaturation, since

$$\delta \Delta \mu_{2,tt} = \Delta H^D_{2,tt} - T \Delta S^D_{2,tt} - \Delta H^N_{2,tt} + T \Delta S^N_{2,tt}$$  \hspace{1cm} (19)

The analysis described in Fig. 8 has given approximate $\Delta H_{2,tt}$ and $\Delta S_{2,tt}$ values for the native protein. For denatured protein, data are even more scarce. Nevertheless, approximate values for the denatured protein below the transition temperature were generated by extrapolating the $(\partial \mu_2 / \partial m_3)_{T,P,m_2}$ curve at pH 2.8 to the low temperature zone. This extrapolation was based on two assumptions: 1) that $(\partial m_3 / \partial m_2)_{T,P,m_3}^N - (\partial m_3 / \partial m_2)_{T,P,m_3}^D$ is independent of temperature, and 2) that, in the transition region, the preferential interactions are the weighted average of the parameters for the native and denatured states of the protein, i.e.

$$\left( \frac{\partial \mu_2}{\partial m_3} \right)_{T,P,m_3}^N = f_N \left( \frac{\partial \mu_2}{\partial m_3} \right)_{T,P,m_3}^N + f_D \left( \frac{\partial \mu_2}{\partial m_3} \right)_{T,P,m_3}^D$$  \hspace{1cm} (20)

where $f_N$ and $f_D$ are the fractions of the protein in the native and denatured states. The resulting calculated temperature dependence of $(\partial \mu_2 / \partial m_3)_{T,P,m_3}$ for the denatured protein below the transition is shown by the dotted line of Fig. 7. Construction of a Van't Hoff plot, given in Fig. 10A and application of Eq. (8) permitted the calculation of the temperature dependence of the partial molal enthalpy and entropy variations with trehalose concentration. The results are plotted in Fig. 10B and C. Both parameters are seen to increase with temperature, as had been found for the native protein. While the highly approximate nature of these calculations requires that the resulting numbers be considered only as estimates, nevertheless it would seem that the thermodynamic parameters for denatured RNase A follow a dependence on

![Fig. 10. Temperature dependence of the variation with trehalose concentration of the calculated transfer free energy (A), transfer enthalpy (B) and transfer entropy (C) of denatured RNase A in 0.5 M aqueous trehalose solution.](image-url)
Table 7
Transfer enthalpies of native and denatured RNase A at 52°C in aqueous trehalose solutions

<table>
<thead>
<tr>
<th>Trehalose/M</th>
<th>m₁/(mol/1000 g H₂O)</th>
<th>Δ Hₚ⁰ - Δ Hₚ o</th>
<th>Δ H₂ₓ,n</th>
<th>Δ H₂ₓ,d</th>
<th>(Δ Δ H₂ₓ - 2Δ H₂ₓ, r,P,m₁) / (Δ / °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.209</td>
<td>0.1 (0.8)</td>
<td>23.3</td>
<td>23.4 (24.1)</td>
<td>127.4 (140.6)</td>
</tr>
<tr>
<td>0.3</td>
<td>0.320</td>
<td>-0.6 (1.2)</td>
<td>39.6</td>
<td>39.0 (40.8)</td>
<td>147.4 (161.9)</td>
</tr>
<tr>
<td>0.4</td>
<td>0.436</td>
<td>-2.4 (1.8)</td>
<td>59.7</td>
<td>56.8 (61.0)</td>
<td>168.3 (184.1)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.558</td>
<td>-3.3 (2.5)</td>
<td>82.3</td>
<td>79.0 (84.8)</td>
<td>190.3 (207.4)</td>
</tr>
</tbody>
</table>

\( ^a \text{kcal mol protein}^{-1}. \)
\( ^b \text{kcal mol protein}^{-1} \text{mol trehalose}^{-1}. \)
\( ^c \) The numbers listed are at pH 5.5; numbers in parentheses are at pH 2.8.

trehalose concentration that is qualitatively similar to that of the native protein.

Finally, the availability of these values, even though estimated and therefore highly approximate, has permitted us to evaluate the magnitude of the contribution of interactions with the co-solvent to the measured standard enthalpy and entropy of denaturation. This contribution can be expressed by relations similar to Eq. (18) for the free energy. For the standard enthalpy change, the increment, \( \delta \Delta H^o \), is given by

\[
\delta \Delta H^o = \Delta H^o_{m_1} - \Delta H^o_w = \Delta H^D_{2,ur} - \Delta H^N_{2,ur}
\]

(21)

The calculations are summarized in Table 7, where \( \Delta H^D_{2,ur} \) was calculated from \( (\Delta H^o_{m_1} - \Delta H^o_w + \Delta H^N_{2,ur}) \) (Eq. (21)), using the data of Table 2 and Fig. 8D. The corresponding values of the slope, e.g. 199 ± 10 kcal mol protein⁻¹ mol trehalose⁻¹ at 0.5 M trehalose, agree well with those calculated from the extrapolated values of \( (\partial \mu_r / \partial T)_{\gamma,P,m_1} \) namely 196 kcal mol protein⁻¹ mol trehalose⁻¹, presented in Fig. 10B.

Examination of the values of the various measured and calculated (even though very approximately) parameters leads to the observations that: 1) the increments of the transfer free energy during denaturation are of the same magnitude as the values of \( \Delta \mu_{2,ur} \) of the native or denatured proteins; for example, at 52°C, 0.5 M trehalose, \( \Delta \mu_{2,ur} \) has values of -0.73 and -0.20 kcal (mol protein)⁻¹ (mol trehalose)⁻¹ at pH 5.5 and 2.8, respectively, and a stabilizing contribution (\( \delta \Delta G^o \)) of 1.0 ± 0.3 kcal mol⁻¹. 2) The corresponding enthalpic contributions, however, differ greatly. At the same condition, \( \Delta H^D_{2,ur} \) has a value of 82.3 kcal (mol protein)⁻¹ (mol trehalose)⁻¹ and \( \Delta H^o \) of denaturation has values 85.3 and 105.6 kcal (mol protein)⁻¹ (mol trehalose)⁻¹ at pH 2.8 and 5.5 respectively. The enthalpy increment during denaturation, however, is very small, \( \delta \Delta H^o = 0 ± 3 \) kcal mol⁻¹. This leads to the conclusions that: 1) The very small free energy values result from frequently large enthalpies that must be close to compensated by equally large entropic contributions, which for the transfer parameters are shown in Fig. 8. 2) The enthalpic contributions of interactions with the co-solvent system to the stabilization during denaturation are very small relative to the standard enthalpy change in the absence of the stabilizer, as well as to the absolute values of the transfer enthalpies. This means that, on denaturation, the interactions with the newly exposed structural elements of the protein must be similar in nature to those with the surface of the native protein. This is consistent with recent analysis by Liu and Bolen [57] of the interactions of osmolytes with amino acid side chains and peptide groups. These authors have shown that sucrose and sarcosine have a weak affinity for both polar and some non-polar residues, but a lower affinity than water for peptide groups. Since protein unfolding exposes to contact with solvent a large number of previously buried peptide groups, this report leads to the anticipation that, in the presence of osmolytes, preferential interaction should be less positive (lower binding or
higher exclusion) with unfolded protein than with its native form. This is exactly the observation made in the present study and permits the reduction in preferential binding on unfolding to be ascribed to the exposure of peptide groups previously not available to solvent molecules.

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References