Effect of D-amino acids on the functional activity and conformational stability of ribonuclease-A

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Received 22 April 2002; revised 2 July 2002

Using cytidine 2':3' cyclic monophosphate as a substrate, $K_{m}$ and $k_{cat}$ of ribonuclease-A in the presence of different concentrations of D-amino acids (Ala, Ser, Pro and Lys) and their L-isomers were measured at pH 6.0 and 25°C. These kinetic parameters remained unchanged in the presence and absence of D- and L-amino acids. This is the first experimental evidence showing that D-amino acids are compatible with the enzyme function. Values of $T_m$ (midpoint of denaturation), $\Delta H_m$ (enthalpy change at $T_m$) and $\Delta C_p$ (constant-pressure heat capacity change) were also determined from the heat-induced denaturation curves of the protein, measured in the presence and absence of D- and L-isomers of an amino acid at four different pH values. It is shown for the first time that these thermodynamic parameters, within experimental errors, do not depend on the stereospecificity of an amino acid. Estimates of $\Delta G^0$ with the help of Gibbs-Helmoltz equation ($\Delta G^0 = \Delta H_m - T \Delta C_p$) we determined using known values of $T_m$, $\Delta H_m$ and $\Delta C_p$, suggested that D- and L-amino acids are compatible with protein stability, for $\Delta G^0$ remained unchanged in the presence of amino acids.

It has long been believed that only L-forms of amino acids occur in nature, but D-amino acids, although stereotyped as "unnatural", are also widely distributed in living organisms. For instance, D-Ser, D-Ala and D-Pro have been found in human brain and plasma (refs. 3, 4 and refs. therein). Free D-Ser and D-Asp have been identified in other mammals, besides human systems. Furthermore, protein-bound D-Asp and D-Glu and other D-amino acids have been found in various animal tissues including those of human (ref. 7 and refs. therein). Some hyperthermophilic archaebacteria also contain appreciably high amounts of free D-amino acids. D-Amino acids incorporated in the polypeptide backbone are important components of eubacterial cell walls. Antibiotics produced by many micro-organisms also contain D-amino acids.

The externally supplied free D-amino acids, if not metabolized by D-amino acid oxidase and D-aspartate oxidase can result in the inhibition of metabolic activity or growth of animals. This is quite surprising, for free D-amino acids are known to be present in the internal environment of various cells. This prompted us to carry out in vitro studies of the effects that free D-amino acids may have on the functional activity and stability of enzymes. This is the first report that looks at the effects of D-amino acids (Ala, Ser, Pro and Lys) on the functional activity and stability of a model protein, ribonuclease-A (RNase-A); for the purpose of comparison, L-isomers of these amino acids have also been studied.

We report that (a), D-amino acids are compatible with the enzyme function, for $K_{m}$ (Michaelis constant) and $k_{cat}$ (catalytic constant) of RNase-A remain unchanged in the presence and absence of D-amino acids, and (b), both D- and L-amino acids have comparable effects on the conformational stability parameters, $T_m$ (midpoint of denaturation), $\Delta H_m$ (enthalpy change at $T_m$), $\Delta C_p$ (constant-pressure heat capacity change) and $\Delta G^0$ (Gibbs energy change at 25°C).

Materials and Methods

Lyophilized bovine pancreatic RNase-A (Type III-A, Lot # 7747009) and cytidine 2':3' cyclic monophosphate (Lot # 4147226) were purchased from Sigma Chemical Company, USA, and D- and L-forms of alanine, serine, lysine and proline were obtained from Aldrich Chemical Co. Ltd. Electrophoretic homogeneity of the commercial
sample of RNase-A was checked on SDS-PAGE. It
gave one band on the gel. This protein and other
chemicals, which were analytical grade reagents, were
used without further purification.

RNase-A stock solution was prepared by dialyzing
against several changes of 0.1 M KCl solution at
4°C. The dialyzed protein was then filtered using 0.45
μm Millipore filter paper. Protein concentration was
determined using a value of 9800 M⁻¹ cm⁻¹ for the
molar absorption coefficient (ε) at 277.5 nm. All
solutions used for optical measurements were
prepared in the desired degassed buffer containing 0.1
M KCl. The buffer solutions were used 0.05 M citrate
buffer for the pH values 5.0 and 4.5, and 0.05 M
glycine-HCl buffer for the pH values 3.1 and 2.5.

For activity measurements all solutions were
prepared in the degassed 0.05 M citrate-0.1 M KCl
buffer of pH 6.0. RNase-A activity using cytidine 2'–
3' cyclic monophosphate (C₃p) as substrate was
assayed as described by Crook et al. For the control
experiment (in the absence of an amino acid) required
amount of the substrate was placed in both the sample
and reference cuvettes placed in Shimadzu UV-Vis
double beam spectrophotometer and the temperature
maintained at 25 ± 0.5°C. In order to follow the
progress curve for the RNase-A mediated hydrolysis
of C₃p, required amount of the enzyme was added to
the sample cuvette by rapid mixing only after adding
the same volume of the buffer as that of the protein
solution to the blank cuvette. The continuous
recording of the change in the absorbance at 292 nm
was done for 20 min. Each progress curve
(absorbance change at 292 nm versus time) obtained at
different substrate concentrations was analyzed for the
initial velocity (v) at a given substrate concentration
[S] as described by Crook et al. In order to obtain
values of Kₚ and kcat of the enzyme, (v, [S]) data were
fitted to the relation, v = kcat × [S] / (Kₚ + [S]) using
the least-squares method. For activity measurements
in the presence of an amino acid, three stock solutions
namely enzyme, substrate and buffer, all containing
same amount of the amino acid were prepared.
Progress curves for the RNase-A mediated hydrolysis
of C₃p in the presence of different concentrations of
an amino acid were followed for 20 min. These
observations were used for the determination of v, Kₚ
and kcat by the same method as used in the case of the
control experiments.

Thermal denaturation measurements in the
temperature range 20-85°C were carried out in Jasco
V-560 UV/Vis spectrophotometer with a heating rate
of 1°C/min using a Peltier type temperature controller
(ETC-505T). Heat-induced denaturation curves of
RNase-A in the presence and absence of various
amino acids at different pH values were measured by
observing changes in the absorption of the protein at
287 nm. Reversibility of the protein denaturation in
the presence and absence of amino acids was checked
by cooling the sample from 85 to 20°C and then
comparing its optical property with that of the
unheated native protein. About 380 data points were
collected for each denaturation curve.

Using a non-linear analysis, values of T_m and ΔH_m
associated with each denaturation curve [i.e., ΔE₃₉₇
(difference in the values of E₃₉₇ between T° and
20°C)] versus T were obtained as described earlier.
This procedure involves fitting the entire (ΔE₃₉₇. T)
data of each denaturation curve to the relation:

\[ y(T) = \frac{y_N(T) + y_D(T)\exp[-\Delta H_m/R (1/T - 1/T_m)]]}{\left[1 + \exp[-\Delta H_m/R (1/T - 1/T_m)]\right]^2} \]  \hspace{1cm} (1)

where, y(T) is the experimentally observed ΔE₃₉₇ of
the protein at temperature T K, y_N(T) and y_D(T) are
the values of ΔE₃₉₇ of the native and denatured protein
molecules at T K, respectively, and R is the gas
constant. The parabolic model for the temperature-
dependencies of the baselines (i.e., y_N(T) = a_N + b_N T +
c_N T^2, and y_D(T) = a_D + b_D T + c_D T^2) where a, b and c
are temperature-independent coefficients and subscripts N and D refer to the native and denatured
molecules, respectively) was used in the analysis of
each transition curve.

Values of ΔG_D at different temperatures in the
transition region were estimated using the relation:

\[ \Delta G_D(T) = -RT \ln \left[ \frac{y(T) - y_N(T)}{y_D(T) - y(T)} \right] \]  \hspace{1cm} (2)

where y has the same meaning as in equation (1).

Values of ΔC_p in the presence and absence of
various amino acids were obtained from the linear
least-squares analysis of the ΔH_m versus T_m plot. The
value of ΔG_D(T) was estimated with the help of the
following Gibbs-Helmoltz equation:

\[ \Delta G_D(T) = \Delta H_m \left( \frac{T_m - T}{T_m} \right) - \Delta C_p \left( \frac{T_m - T}{T_m} \right) + T \ln \left( \frac{T_m}{T} \right) \]  \hspace{1cm} (3)
Results

Steady-state kinetic measurements of RNase-A mediated hydrolysis of C\textsuperscript{\textsuperscript{+}}p in the concentration range 0.05-0.5 mg mL\textsuperscript{-1} in 0.05 M citrate-0.1 M KCl, pH 6.0 at 25°C were followed by observing changes in the absorption at 292 nm for 20 min (results not shown). These observations in the presence and absence of an amino acid were used to obtain $K_m$ and $k_{cat}$ values (Table 1) as described in 'Materials and Methods'.

Heat-induced denaturation curves of RNase-A in the presence of different concentrations (0, 0.25, 0.50, 0.75 and 1.00 M) of D-amino acids (Ala, Ser, Pro and Lys) and their stereoisomers were measured by following changes in $\Delta_{287}$ as a function of temperature at four different pH values (5.0, 4.5, 3.1 and 2.5). It has been observed that (a), the temperature-dependence of $y_n$ ($\Delta_{287}$ of the native protein) does not depend on the type and concentration of an amino acid at all pH values.

Table 1—Kinetic parameters of RNase-A in the presence of D- and L-amino acids at pH 6.0 and 25°C.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (min\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>1.35±0.15</td>
<td>125±7</td>
</tr>
<tr>
<td>0.10</td>
<td>1.29±0.10</td>
<td>120±6</td>
</tr>
<tr>
<td>0.50</td>
<td>1.42±0.15</td>
<td>124±5</td>
</tr>
<tr>
<td>Pro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>1.35±0.15</td>
<td>125±7</td>
</tr>
<tr>
<td>0.10</td>
<td>1.45±0.21</td>
<td>115±10</td>
</tr>
<tr>
<td>0.50</td>
<td>1.23±0.14</td>
<td>109±7</td>
</tr>
<tr>
<td>Ser</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>1.35±0.15</td>
<td>125±7</td>
</tr>
<tr>
<td>0.10</td>
<td>1.25±0.17</td>
<td>126±8</td>
</tr>
<tr>
<td>0.50</td>
<td>1.34±0.18</td>
<td>111±8</td>
</tr>
<tr>
<td>Lys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>1.35±0.15</td>
<td>125±7</td>
</tr>
<tr>
<td>0.10</td>
<td>1.38±0.19</td>
<td>117±9</td>
</tr>
<tr>
<td>0.50</td>
<td>1.24±0.18</td>
<td>114±8</td>
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</table>

Typical denaturation profiles of RNase-A in the presence of different concentrations of D- and L-amino acids at a constant pH are shown in Figs. 1A and 2A, respectively. Assuming that the heat-induced denaturation in the presence of an amino acid is also of a two-state type, each transition curve at a fixed pH and [amino acid], the molar amino acid concentration was analyzed according to equation (1) with three fixed parameters, namely, $a_n = -5259$, $b_n = 35.56$, $c_n = -6.007 \times 10^2$, and five free parameters, namely, $a_D$, $b_D$, $c_D$, $\Delta H_m$ and $T_m$. Values of $\Delta H_m$ and $T_m$ in the presence of different concentrations of various amino acids are shown in Fig. 3.

A plot of $\Delta H_m$ versus the corresponding $T_m$ in the absence and presence of an amino acid at a given concentration (e.g., see Figs. 1B and 2B) was used to obtain $\Delta C_p$ using the linear least-squares procedure. Values of $\Delta C_p$ in the absence and presence of different concentrations of D- and L-amino acids are given in Table 2.

Theoretical stability curves of RNase-A were constructed using equation (3) with known values of $\Delta H_m$, $T_m$ and $\Delta C_p$ in a given solvent condition. Figs. 1C and 2C show typical stability curves of RNase-A in the presence of D- and L-amino acids. At a given [amino acid], values of $\Delta G_0$ in the range $-5.4 \leq \Delta G_0 \leq 5.4$ kJ mol\textsuperscript{-1} are estimated at different temperatures with the help of equation (2) from the corresponding.
Fig. 1—(A): Thermal denaturation of RNase-A in the presence of different concentrations of D-Ala at pH 5.0 (0.05 M citrate-0.1 M KCl buffer) [(O), 0 M; (A), 0.5 M; and (□), 1.0 M. For the sake of clarity, all data points are not shown, and for the same reason transition curves at 0.25 and 0.75 M of the amino acid have been omitted. The inset shows thermal denaturation curves in the absence of amino acids at different pH values] (B): Plots of denaturational enthalpy versus temperature in the presence of different concentrations of D-Ala [For the sake of clarity plots in the absence and presence of 0.75 M D-Ala are not shown] (C): Stability curves of the protein in the presence and absence of 1.0 M D-Ala [ΔGΔ values in the range -5.4 ≤ ΔGΔ, kJ mol⁻¹ ≤ 5.4 were determined from the measured denaturation curves at different temperatures using equation (2), and these are represented by open circles. For the sake of clarity all (ΔGΔ, T) data points and stability curves at other Ala concentrations are not shown. The solid lines were drawn according to equation (3) with corresponding values of ΔHα and Tα given in Fig. 3 and ΔCα values given in Table 2. The error bars represent errors in ΔGΔ(T) values due to errors in ΔHα and ΔCα (see Text)].
Fig. 2.—(A): Thermal denaturation of RNase-A in the presence of different concentrations of L-Ala at pH 5.0 (0.05 M citrate-0.1 M KCl buffer) [Symbols have the same meaning as in Fig. 1A, except that data are for l-Ala] (B): Symbols have the same meaning as in Fig. 1B, except that results are for L-Ala (C): Stability curves of the protein in the presence and absence of 1.0 M l-Ala [Open circles have the same meaning as in Fig. 1C. For the sake of clarity all (ΔGp, T) data points and stability curves at other l-Ala concentrations are not shown. Solid lines were drawn according to equation (3) using corresponding values of ΔHn and Tn given in Fig. 3 and ΔCp given in Table 2. Errors shown in ΔGp(T) are due to errors in ΔHn and ΔCp (see Text)]

Table 2—Values of temperature-dependence of the enthalpy change of RNase-A in the presence of different concentrations of D- and L-amino acids

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Ala</th>
<th>Ser</th>
<th>Pro</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>(M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>5.06±0.34</td>
<td>5.06±0.34</td>
<td>5.06±0.34</td>
<td>5.06±0.34</td>
</tr>
<tr>
<td>0.25</td>
<td>5.43±0.31</td>
<td>5.18±0.74</td>
<td>5.20±0.70</td>
<td>5.23±0.56</td>
</tr>
<tr>
<td>(0.31±0.63)</td>
<td>(5.39±0.44)</td>
<td>(5.49±0.57)</td>
<td>(5.69±0.55)</td>
<td>(5.73±0.51)</td>
</tr>
<tr>
<td>0.50</td>
<td>5.37±0.31</td>
<td>5.22±0.63</td>
<td>5.09±0.55</td>
<td>5.36±0.49</td>
</tr>
<tr>
<td>(0.56±0.76)</td>
<td>(5.08±0.51)</td>
<td>(5.44±0.45)</td>
<td>(5.80±0.48)</td>
<td>(5.85±0.62)</td>
</tr>
<tr>
<td>0.75</td>
<td>5.61±0.43</td>
<td>5.53±0.34</td>
<td>5.35±0.54</td>
<td>5.36±0.19</td>
</tr>
<tr>
<td>(0.13±0.29)</td>
<td>(0.01±0.42)</td>
<td>(5.35±0.52)</td>
<td>(5.85±0.62)</td>
<td>(5.85±0.62)</td>
</tr>
<tr>
<td>1.00</td>
<td>5.32±0.23</td>
<td>5.36±0.46</td>
<td>5.42±0.41</td>
<td>5.30±0.41</td>
</tr>
<tr>
<td>(0.72±0.35)</td>
<td>(0.56±0.40)</td>
<td>(5.40±0.20)</td>
<td>(5.75±0.51)</td>
<td>(5.75±0.51)</td>
</tr>
</tbody>
</table>
Fig. 3—(A): $\Delta H_m$, dependence of RNase-A on various D- and L-amino acid concentrations at four different pH values (5.0, 4.5, 3.1 and 2.5). [O], in the absence of amino acids; (Δ), D-Ala; (□), L-Ala; (V), D-Ser; (■), L-Ser; (●), D-Pro; (+), L-Pro; (●), D-Lys; (●), L-Lys) (B): Dependence of $T_m$ of RNase-A on various D- and L-amino acid concentrations at four different pH values (5.0, 4.5, 3.1 and 2.5) [Symbols have the same meaning as in A].
heat-induced denaturation curve, and are shown on the stability curves (Figs. 1C and 2C).

**Discussion**

The activity measurements of the RNase-A mediated hydrolysis of C\textsubscript{34}p in the absence and presence of amino acids at pH 6.0 at 25°C show that the values of \( K_m \) and \( k_{cat} \) (Table 1) depend neither on the stereospecificity nor on the concentration of amino acids. These are expected results at least for L-isomers, for Timasheff and co-workers\(^{14-16} \) have shown that L-amino acids are preferentially excluded from the protein domain, hence should have no effect on the enzymatic activity. These observations are also in agreement with the earlier findings that L-amino acids have no significant effect on the functional activity of enzymes\(^{17,18} \). It is interesting to note that the non-chiral amino acids (Gly) and modified amino acids, e.g., glycine derivatives and \( \alpha \)- and \( \beta \)-amino acids are preferentially excluded from the protein domain\(^{14-16} \). These findings suggest that preferential exclusion of amino acids from the protein domain would not depend on the stereospecificity of amino acids. If this is the case, it is then expected that the kinetic properties of RNase-A in the presence of D-amino acid should be the same as that of the L-amino acids. Our results shown in Table 1 are in agreement with this expectation and suggest that, contrary to the earlier suggestion\(^7 \), D-amino acids will not affect the metabolic functions of enzymes.

A two-state mechanism was assumed in the estimation of \( \Delta H_m \) and \( T_m \) from the heat-induced denaturation curves of RNase-A (e.g., see Figs. 1A and 2A) using the procedure described earlier\(^{20} \). A few comments are therefore necessary. There exists calorimetric evidence that RNase-A denaturation in the absence of an amino acid is of the two-state type\(^9,22 \). On the other hand, no such data are available for validating the two-state assumption used in the analysis of the heat-induced denaturation curves of this enzyme in the presence of amino acids used in this study. However, a two-state assumption may not be a bad one in these cases as well. There are at least three reasons for saying this: (a), the heat-induced denaturation curves of RNase-A in the presence of amino acids are as symmetrical as the one observed in their absence (e.g., see Figs. 1A and 2A); (b), DSC measurements have shown that thermal denaturation of RNase-A in the presence of glycine and glycine based osmolytes follows a two-state mechanism\(^9,21 \); and (c), thermal denaturation of other proteins in the presence of amino acids follows a two-state mechanism\(^{20,21} \).

At each fixed pH, \( \Delta H_m \) of RNase-A within experimental errors, remains unchanged in the presence of various concentrations of D- and L-amino acids (Fig. 3A), whereas \( T_m \) increases with an increase in D- and L-amino acid concentration (Fig. 3B). These findings are in agreement with the earlier observations on RNase-A in the presence of Gly and its derivatives\(^{20} \). Our observations on \( \Delta H_m \) and \( T_m \) at a fixed pH seem to suggest that \( \Delta C_p \) of the protein in the presence of amino acids is zero, for \( \Delta C_p \) is equal to the derivative, \( \Delta H_m / \Delta T_m \) (see also ref. 20). In order to see whether this is a correct observation for a given [amino acid], we have plotted values of \( \Delta H_m \) measured at different pH values versus corresponding \( T_m \) values (e.g., see Figs. 1B and 2B). A linear least-squares analysis of such a plot gives the value of \( \Delta C_p \) (see Table 2). It is seen in Table 2 that the value of \( \Delta C_p \) of RNase-A in the absence of amino acids is 5.06 \( \pm \) 0.34 kJ mol\(^{-1} \) K\(^{-1} \), which is in excellent agreement with the calorimetric \( \Delta C_p \) (5.14-5.30 kJ mol\(^{-1} \) K\(^{-1} \)) reported earlier\(^{21,22} \). This agreement led us to believe that our measurements of transition curves and their analysis for thermodynamic parameters are authentic and accurate. It is also seen in Table 2 that \( \Delta C_p \) of RNase-A increases with an increase in [amino acid]. This observation is in agreement with the finding that \( \Delta C_p \) of RNase-A increases with an increase in sarcosine concentration\(^{20} \).

It is seen in Fig. 3B that at a given pH, \( T_m \) of RNase-A increases with an increase in D- and L-amino acid concentrations. The finding that the protein is stabilized in terms of \( T_m \) by L-amino acids agrees with those reported earlier\(^{15,20,21,24,25} \). In order to explain this observation on \( T_m \), two mechanisms of stabilization of proteins by naturally occurring osmolytes (L-amino acids) were proposed\(^{14-16,20} \). According to Timasheff and co-workers\(^{14-16} \), the main factor for the stabilization effect of osmolytes such as L-amino acids on the protein is related to the fact that these osmolytes cause preferential hydration of the protein; that is, this effect should favor a protein state with lower exposed surface, thus displacing the denaturation equilibrium, N conformation \( \leftrightarrow \) D conformation, towards the N state, bringing about a stabilization of the native protein in the presence of osmolytes. Bolen and co-workers\(^{20,25} \) have interpreted the effect of L-amino acid osmolytes on proteins in terms of transfer-free energy of protein groups from water to osmolyte solutions and in terms of
dimensions of the native and denatured protein molecules in osmolyte solutions. They concluded that,
in addition to raising the overall $\Delta G_{D}$, the transfer-free
energy ($\Delta G_{D} = \Delta G_{D(N)} + \Delta G_{D(N)}$), the unfavorable interaction of the protein backbone with osmolyte
causes a collateral effect that results in displacing the
denaturation equilibrium towards the N state, and
bringing about a stabilization of N conformation in
the presence of osmolytes. The observation that $T_m$ of
RNase-A increases with an increase in D-amino acid
concentration (see Fig. 3B) leads us to believe that the
mechanism of stabilization of protein by both D- and
L-isomers is the same.

In order to see how amino acids affect $\Delta G_{D}$ of
RNase-A at different temperatures, $\Delta G_{D}$ values in a
given solvent condition were estimated in the
temperature range 0-100°C using equation (3) with
known values of $\Delta H_m$ and $T_m$ (Fig. 3) and $\Delta C_P$
(Table 2), and stability curves were constructed.
Typical stability curves are shown in panel C of
Figs. 1 and 2. It may be noted that the errors in the
estimation of $\Delta G_{D}$ at a given temperature are due to
errors in the measurements of $\Delta H_m$ and $\Delta C_P$. A few
comments are, however, necessary: (i), the value of $\Delta G_{D}(T)$ at a given $pH$ is obtained using equation (3)
with all the best fitting parameters. For example, a
value of 39.9 kJ mol$^{-1}$ for $\Delta G_{D}$ of the protein in the absence of an amino acid at $pH$ 5.0 is
obtained using the best fitted parameters, $\Delta H_m = 443$ kJ mol$^{-1}$, $T_m = 338.0$ K and $\Delta C_P = 5.06$ kJ mol$^{-1}$ K$^{-1}$;
(ii), following Becktel and Schellman$^{29}$, we have
obtained upper and lower values of $\Delta G_{D}$ using equation (3) with the upper and lower limits of standard
errors in the measurements of $\Delta H_m$ which is
$\pm 13$ kJ mol$^{-1}$ and $\Delta C_P$ which is $\pm 0.34$ kJ mol$^{-1}$ K$^{-1}$.

For example, the upper value of $\Delta G_{D}$ is (42.2 kJ mol$^{-1}$) of RNase-A in the absence of an amino acid at
$pH$ 5.0 is obtained using $\Delta H_m = 443 + 13 = 456$ kJ
mol$^{-1}$ and $T_m = 338.0$ K and $\Delta C_P = 5.06 - 0.34 = 4.72$
kJ mol$^{-1}$ K$^{-1}$ and the lower value of $\Delta G_{D}$ is (37.6 kJ
mol$^{-1}$ is obtained using $\Delta H_m = 443 - 13 = 430$ kJ
mol$^{-1}$, $T_m = 338.0$ K and $\Delta C_P = 5.06 + 0.34 = 5.40$ kJ
mol$^{-1}$ K$^{-1}$. The error in each $\Delta G_{D}$ value shown in Figs.
1C and 2C represents deviation from the mean of the
upper and lower values of $\Delta G_{D}$, which, for instance, is
$\pm 2.3$ kJ mol$^{-1}$ for $\Delta G_{D}$ of the protein in the absence of an amino acids at $pH$ 5.0. Finally, we did not
consider the effect of the error in the determination of
$T_m$ on $\Delta G_{D}$, for in our study it has insignificant effect
on $\Delta G_{D}$.

Several interesting observations can be made from
the results presented in Figs. 1C and 2C: (a), the error
in the measurements of $\Delta G_{D}$ in the transition region
(i.e., near $T_m$) is very small, whereas it increases at
temperatures below $T_m$. This means that the accuracy
of the extrapolated $\Delta G_{D}$ at a given solvent condition
declines with an increase in ($T_m - T$); (b), there is a
significant increase in the stability in terms of $\Delta G_{D}$ of
the protein in the presence of an amino acid in the
transition zone. This observation is consistent with the
prediction of the mechanisms of stabilization described above; (c), amino acids have no significant
effect on $\Delta G_{D}$ in the pretransition region. For
instance, $\Delta G_{D}$ (the value of $\Delta G_{D}$ at 25°C), within
experimental errors, remains unchanged. The latter
observation is consistent with earlier reports that $\Delta G_{D}$
of proteins are not affected by the presence of 1-
amino acids$^{24,25}$. One possible explanation for the
different effects of amino acids on Gibbs energy at
different temperatures (see Figs. 1C and 2C) is that
the action of the osmolyte is on the denatured
ensembles and not on the native state. A support for
this possibility comes from the works of Bolen and
coworkers$^{26-28}$ who have shown that osmolyte-protein
interaction results in the contraction of the denatured
ensembles (i.e., it promotes the formation of native
molecules), whereas it has no effect on the dimension
of the native folded molecules. Our results also
suggest that effect of D-amino acid on the Gibbs
energy change associated with the process
N conformation $\leftrightarrow$ D conformation is identical to that
of L-amino acid.

Conclusions

D-Amino acids (Ala, Ser, Pro and Lys) do not affect
the functional activity ($K_m$ and $k_{cat}$) of RNase-A under
native conditions. Hence, they are compatible with
enzyme function; also they do not affect $\Delta G_{D}$ of
RNase-A. However, they protect the protein against
heat denaturation. Hence, action of amino acids is on
the denatured protein, but not on the native molecules.
Although $T_m$ of RNase-A is increased in the presence
of D-amino acids, $\Delta H_m$ is independent of the type and
concentration of these amino acids. Hence protein
stabilization by amino acids is under entropic control.

Acknowledgement

This work was supported by grants from the
Department of Science and Technology (SP/SDI-26/96) and Council of Scientific and Industrial
Research [37(976)/98 EMR-II], N Delhi, India.
References