Both the Fast and Slow Refolding Reactions of Ribonuclease A Yield Native Enzyme

(pH jump/stopped flow/inhibitor binding/tyrosine absorbance)

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ABSTRACT The fast reaction (t ~ 50 msec) observed previously in the refolding of thermally unfolded ribonuclease A (disulfide bonds intact) has now been studied by two properties indicative of enzyme function: binding of a competitive inhibitor (2'CMP) and hydrolysis of a substrate (CpA → C > p + A). Both the binding and catalytic reactions are fast (<2 msec) compared to refolding. Binding of 2'CMP occurs during both fast and slow refolding reactions, and the protein folded in the fast reaction has a normal binding constant for 2'CMP. Recovery of enzymatic activity during the fast refolding reaction, as measured by the rate of CpA hydrolysis, parallels the kinetic curve for 2'CMP binding. When the kinetics of refolding are measured by the burying of exposed tyrosine groups, no difference is found. The presence of 2'CMP has no effect on the kinetics of refolding.

The results show that the fast refolding reaction does not yield an intermediate in the refolding of RNase A. Instead, both fast and slow refolding reactions have a common product, fully active RNase A. Although they show a 100-fold difference in rates of refolding, the starting materials for the fast and slow refolding reactions have similar properties, as regards: (a) the molar absorbancy at 286 nm, reflecting the state of exposed tyrosine groups, and (b) their apparent failure to bind 2'CMP.

Both fast and slow refolding reactions have been observed in studies of the reversible unfolding transitions of a few simple small proteins: in the pH-induced refolding of staphylococcal nuclease (1) and bovine pancreatic ribonuclease A (2) and in the solvent-induced refolding of horse-heart ferri-cytochrome c (3, 4) and chicken egg-white lysozyme (5) in guanidinium solutions. The interpretation of these fast refolding reactions has been in doubt, but it has been supposed that they represent the formation of one or more intermediates in refolding; either intermediates on the normal pathway (1) or abortive intermediates, not on the direct pathway (3, 4). An alternative possibility is that the fast reactions represent the conversion of intermediates to final product. For RNase A, this may be tested by measuring the enzymatic activity of the product of fast refolding in a time range where the extent of slow refolding is small.

In previous work (2), the refolding of RNase A has been measured by the changes in absorbance at 286 nm or at 240 nm that result chiefly from shielding tyrosine groups from solvent. There are six tyrosine groups in RNase A. They are found in different regions of the three-dimensional structure (6, 7) rather remote from the active site, and all of the tyrosine groups are at least partly shielded from water at neutral pH, where they are not ionized (7). Titration experiments (8, 9) show that three of the six tyrosine groups are not free to ionize normally in native RNase A, and spectral studies (10, 11) also show differences between individual tyrosine groups. Spectral changes caused by burying tyrosine groups during refolding probably reflect conformational changes in more than one region of the RNase A molecule. Partial refolding might be observable via the tyrosine groups, whereas substrate binding and catalytic activity are likely to require complete refolding.

EXPERIMENTAL

Materials. (a) RNase A: Worthington lot no. RASE 2HB, filtered through Sephadex G-25 (Pharmacia) to remove phenol and phosphate; stored in 0.1 M NaClO₄ concentration measured by absorbance at 278 nm, neutral pH, using a molar absorbance of 9.8 × 10⁴ (20). (b) 2'CMP: P. L. Biochemicals lot 273-10; concentration measured using a molar absorbance of 7.6 × 10⁴ at 260 nm, neutral pH (21); the ratio A₂₅₀/A₂₈₀ was found to be 0.84 [lit. 0.85 (21)]. (c) CpA: Sigma lot 30 G-7490; molar absorbance (266 nm) = 2.1 × 10⁴ in 0.1 N HCl (22).

Buffers. (a) pH 5.8: 0.1 M NaClO₄, 0.05 M cacodylate. (b) pH 2.0: 0.1 M NaClO₄, sufficient HClO₄ to give pH 2.0.

Methods. (a) Equilibrium measurements of absorbance were made in a Cary 14 spectrophotometer with a water-jacketed cell holder; the temperature was measured by a dipping thermistor in the cell. (b) Kinetic measurements were made with a modified Gibson-Durrum stopped-flow instrument, using a Tektronix 564 storage oscilloscope. The temperature was controlled by water circulation. After degassing, solutions were transferred to the driving syringes by plastic disposable syringes (Stylex) but were not allowed to stay in contact with plastic for more than a few minutes. Long storage (30 min or more) of RNase A (pH 2.0) in the plastic syringes gave rise to new, slower, refolding reactions in which the refolded protein was enzymatically inactive.

RESULTS

Refolding of thermally unfolded RNase A goes to completion after a pH jump from 2.0 to 5.8. There is a substantial change in A₂₅₀ when 2'CMP binds to native RNase A (12). [The change is believed to occur in the spectrum of the nucleotide, not that of the enzyme (13).] Binding is rapid (k = 1 × 10⁶ M⁻¹ sec⁻¹ at 15°C, pH 6 (14)] and a specific 1:1 complex is formed between 2'CMP and native RNase A (12, 15). We find no difference spectrum when 2'CMP is added to thermally

Abbreviations: RNase A, bovine pancreatic ribonuclease A; 2'CMP, cytidine 2'-phosphate; C > p, cytidine 2',3'-cyclic phosphate; Tₚ, temperature midpoint of the thermal unfolding transition.
TABLE 1. Test for complete refolding of RNase A by 2'CMP binding

<table>
<thead>
<tr>
<th>Experiment</th>
<th>( \Delta A_{280} )†</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Native RNase A (47°, pH 5.8 + 2'CMP, 10 min)</td>
<td>0.185 ± 0.002</td>
</tr>
<tr>
<td>(2) Unfolded RNase A (47°, pH 2.0, 10 min) → Native RNase A (47°, pH 5.8 + 2'CMP, 10 min)</td>
<td>0.181 ± 0.002</td>
</tr>
</tbody>
</table>

* Final concentrations: RNase A, 0.90 \( \times 10^{-4} \) M; 2'CMP, 1.35 \( \times 10^{-4} \) M.
† \( \Delta A_{280} \) is the difference between the value of \( A_{280} \) read after and before adding 2'CMP; the same amount of 2'CMP is added both to the sample and the blank, and \( A_{280} \) is read after 10 min (at pH 5.8, 47°) in a Cary 14 spectrophotometer.

2'CMP to native RNase A is complete within the stopped-flow deadtime of 2 msec and there are no significant later changes in \( A_{280} \) in any of the time ranges used to study fast and slow refolding (Fig. 2a). The wavelength 250 nm has been chosen because \( \Delta A_{280} \) for refolding, in the absence of 2'CMP, is quite small (Fig. 2b) compared to \( \Delta A_{280} \) for the binding of 2'CMP to native RNase A. Stopped-flow measurements of \( \Delta A_{280} \) during refolding after a pH jump 2.0 → 5.8 + 2'CMP show that binding of 2'CMP occurs during both the fast phase (Fig. 3b) and the slow phase (Fig. 3a) of refolding. The relaxation times and amplitudes are closely comparable to previous values (2) obtained at pH 3.9 and 7.0 by studying tyrosine groups.

Binding of 2'CMP occurs during the fast refolding reaction. As expected from the second-order rate constant, binding of 2'CMP to native RNase A is complete within the stopped-flow deadtime of 2 msec and there are no significant later changes in \( A_{280} \) in any of the time ranges used to study fast and slow refolding (Fig. 2a). The wavelength 250 nm has been chosen because \( \Delta A_{280} \) for refolding, in the absence of 2'CMP, is quite small (Fig. 2b) compared to \( \Delta A_{280} \) for the binding of 2'CMP to native RNase A. Stopped-flow measurements of \( \Delta A_{280} \) during refolding after a pH jump 2.0 → 5.8 + 2'CMP show that binding of 2'CMP occurs during both the fast phase (Fig. 3b) and the slow phase (Fig. 3a) of refolding. The relaxation times and amplitudes are closely comparable to previous values (2) obtained at pH 3.9 and 7.0 by studying tyrosine groups.

Table 2. 2'CMP binding in the fast and slow refolding reactions of RNase A (47°, pH 5.8)

<table>
<thead>
<tr>
<th>2'C MP (10⁻⁴ M)</th>
<th>( \Delta \psi ) (fast) (mV)</th>
<th>( \Delta \psi ) (slow) (mV)</th>
<th>( \alpha )†</th>
<th>( \alpha ) calc‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>&lt;5</td>
<td></td>
<td>0.26</td>
<td>0.148</td>
</tr>
<tr>
<td>0.75</td>
<td>0.164</td>
<td>9</td>
<td>35</td>
<td>0.20</td>
</tr>
<tr>
<td>1.25</td>
<td>0.263</td>
<td>18</td>
<td>65</td>
<td>0.25</td>
</tr>
<tr>
<td>2.50</td>
<td>0.400</td>
<td>26</td>
<td>95</td>
<td>0.30</td>
</tr>
<tr>
<td>3.75</td>
<td>0.573</td>
<td>38</td>
<td>140</td>
<td>0.32</td>
</tr>
<tr>
<td>5.00</td>
<td>0.691</td>
<td>40</td>
<td>150</td>
<td>0.31</td>
</tr>
<tr>
<td>7.50</td>
<td>0.798</td>
<td>42</td>
<td>155</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* Fraction of the RNase A sample binding 2'CMP at equilibrium calculated from a spectrophotometrically measured dissociation constant of 1.3 \( \times 10^{-4} \) M at 47° and the RNase A concentration of 3 \( \times 10^{-4} \) M. † Change in signal at 250 nm resulting from 2'CMP binding in the fast and slow refolding reactions, respectively; total signal 2V. ‡ Fraction of the total 2'CMP bound which is bound in the fast refolding reaction. Calculations for a hypothetical case in which the protein species formed in the fast and slow refolding reactions have 10-fold different binding constants for 2'CMP: the products of slow and fast refolding are assigned dissociation constants of 1.3 \( \times 10^{-4} \) M, and 1.3 \( \times 10^{-5} \) M, respectively, and the value of \( \alpha \) at saturating 2'CMP is chosen to be 0.390 (so that the observed and calculated values of \( \alpha \) agree at the highest concentration of 2'CMP used).
Table 3. Comparison of the kinetics of the fast and slow refolding reactions measured by tyrosine groups (A286) and by 2'CMP binding (A250)

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>λ = 286 nm</th>
<th>λ = 250 nm</th>
<th>ΔA286/ΔA250</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau_r$ (msec)</td>
<td>$\tau_l$ (sec)</td>
<td>$\alpha$</td>
</tr>
<tr>
<td>32</td>
<td>62</td>
<td>14.7</td>
<td>0.20</td>
</tr>
<tr>
<td>32 No 2'CMP</td>
<td>65</td>
<td>14.2</td>
<td>0.22</td>
</tr>
<tr>
<td>36</td>
<td>46</td>
<td>10.9</td>
<td>0.21</td>
</tr>
<tr>
<td>40</td>
<td>41</td>
<td>8.7</td>
<td>0.21</td>
</tr>
<tr>
<td>40 No 2'CMP</td>
<td>48</td>
<td>9.0</td>
<td>0.22</td>
</tr>
<tr>
<td>47</td>
<td>96</td>
<td>8.4</td>
<td>0.20</td>
</tr>
<tr>
<td>47 No 2'CMP</td>
<td>90</td>
<td>8.0</td>
<td>0.21</td>
</tr>
<tr>
<td>51</td>
<td>370</td>
<td>7.8</td>
<td>0.17</td>
</tr>
<tr>
<td>51 No 2'CMP</td>
<td>380</td>
<td>7.6</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Initial conditions: RNase A 9.2 × 10^{-4} M; 0.1 M NaClO4, pH 7.5; 1.0 M cacodylate, 0.1 M NaClO4, pH 5.8.

* An average relaxation time of the fast refolding reaction measured as the weight-average value of 1/$\tau_l$(23). † The relaxation time of the slow refolding reaction measured from a plot of ln [A(∞) - A(t)] versus time. ‡ The relative amplitude of the fast refolding reaction expressed as a fraction of the total absorbance change. The absorbance changes associated with the fast and slow reactions have been found by assuming that each reaction follows a single exponential curve.

RNase A formed by fast refolding has a normal binding constant for 2'CMP. We may determine if the proteins folded in the fast and slow refolding reactions have different binding constants for 2'CMP by measuring the relative amounts of 2'CMP bound as a function of 2'CMP concentration. The results (Table 2) show no difference over a 10-fold range of 2'CMP concentration at 47°C, in which the fraction of RNase A binding 2'CMP at equilibrium varies from 0.164 to 0.795. For comparison, we calculated the expected results when the protein refolded in the fast reaction is assigned a 10-fold lower affinity for 2'CMP than the final equilibrium value. The results (Table 2) show that such a difference in affinity would have been detected readily.

The presence of 2'CMP has no effect on the kinetics of refolding measured by tyrosine groups. Since the difference spectrum between the 2'CMP: RNase A complex (12) and the separated reactants is almost zero at 286 nm, where there is a large change in absorbance on refolding, we can use this wavelength to monitor refolding independently of 2'CMP binding. Measurements of refolding at 286 nm show the same kinetics whether or not 2'CMP is present (Table 3); this is true both of the relaxation times and amplitudes. Consequently it is unlikely that 2'CMP binds to the starting material for either the fast or slow refolding reaction.

The kinetics of refolding are the same when measured by tyrosine groups and by 2'CMP binding. Different kinetics of refolding might be observed by buried tyrosine groups and by 2'CMP binding for either of two reasons: the tyrosine groups occur in different regions of the molecule and also might be expected to change in absorbance upon partial refolding, whereas 2'CMP binds at a single site and might be expected to bind only when refolding is complete. Thus, it is interesting to find that the kinetics are exactly the same when measured by either probe (Table 3). No reaction is found by one probe that is not discerned by the other, and the relative amplitudes are the same for both probes. Moreover, the results show that the molar value of ΔA286 during refolding is the same in the fast and slow refolding reactions.*

* The protein folded in the fast reaction has enzymatic activity. The turnover time for RNase A to hydrolyze Cpa giving C + p + A is fast (0.3 msec at 26°C (16)) compared to both refolding reactions. Thus the corresponding change in A286 can be used as a fast probe of the recovery of enzymatic activity on refolding. (The enzyme concentrations used for these experiments are so low that one can ignore the change in A286 caused by RNase A refolding.) Fig. 4b shows that hydrolysis of Cpa by native RNase A (pH 5.8), 47°C produces a linear change with time for at least 2 sec, at specified concentrations of RNase A and Cpa. In these conditions, the slope is also proportional to enzyme concentration (not shown). When the same stopped-flow experiment is repeated with an aliquot of RNase A that has been allowed to unfold previously at pH 2.0, 47°C, and then to refold after a stopped-flow pH jump: 2.0 → 5.8 + Cpa, the results shown in Fig. 4a are observed. The initial rate of Cpa hydrolysis is small compared to that of the control (Fig. 4b) and the rate increases with time of refolding. The amount of enzymatic activity recovered at different times after refolding is given by the ratio of the activity at any time to the activity at time 0.

* This follows from the fact that ΔA286/ΔA250 has the same value in the fast and slow refolding reactions (Table 3). Since the binding of 2'CMP to RNase A has a 1:1 stoichiometry, we may use ΔA286 and the equilibrium binding constant to determine the number of RNase A molecules refolded in a given reaction; thus ΔA286/ΔA250 is directly related to the molar change in absorbance at 286 nm.

Fig. 3. Binding of 2'CMP occurs during both the fast and slow refolding reactions of RNase A (pH 2.0 → 5.8, 47°C, 250 nm). Final concentrations: RNase A, 0.46 × 10^{-3}M; 2'CMP, 0.75 × 10^{-3}M. Total signal 2.2 V.
CONCLUSIONS

(1) The fast reaction observed in the refolding of thermally unfolded RNase A does not represent the formation of an intermediate in refolding; instead, the product is fully active enzyme. It will be interesting to study other systems for which fast refolding reactions have been observed to see if this is a common phenomenon. (2) As judged by the behavior of tyrosine groups, the starting materials for the fast and slow refolding reactions have similar chromophoric properties, even though they show a 100-fold difference in their rates of refolding. (3) More experiments are needed before we can discuss models to represent these results: in particular, corresponding studies are needed of enzymatic activity during the fast phase of unfolding, and these data are not readily obtained by the techniques used here. However, we will comment briefly on three very simple models. (a) Two pathways of refolding: Ufast → N. Here U is the thermally unfolded protein and N is native RNase. This model can be ruled out because it does not give biphase kinetics (essentially all the molecules refold by the fast pathway). (b) Failure to obtain a completely unfolded starting material, because the temperature isn't high enough: U ⇄ N1 ⇄ N2. Here U and N1 (a partly-folded species) are presumed to preexist in the initial conditions; N1 is an intermediate present only in the thermal unfolding transition zone. This model is ruled out by the failure of N1 to disappear at high temperatures, as discussed in the paragraph above. (c) Presence of two (or more) different unfolded forms: U1 ⇄ U2 ⇄ N. Here U1 and U2 are different fast unfolded species that preexist in the initial conditions at all temperatures. They are linked by a common pathway of refolding, and are separated by a slow step. NMR measurements show a slow interconversion between native and thermally unfolded RNase A as well as what appear to be fast interconversions between native and partly unfolded species (18). Since there are reasons to believe that thermally unfolded RNase A retains some structure (19), the model has some a priori plausibility and it is able to explain most, but not all, of our present results.

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**Enzymatic Activity During Refolding of RNase A**