the two phases in refolding to low urea but is the faster of the two phases for unfolding into high urea. Only after separation of the two phases can the isomerization of proline-93 be accurately determined. In order to explain the apparent discrepancy of isomerization rates during unfolding and refolding, it was proposed that refolding occurred through an enzymatically active structural intermediate I_N (Nall et al., 1978; Cook et al., 1979), which contains one or more prolines in the incorrect isomeric form. Our data rule out the specific suggestion (Schmid, 1981; Schmid & Blaschek, 1981) that it is proline-93 which is in the incorrect form in I_N and in general provide no positive support for the existence of I_N.

In contrast to the CT process, very little is known about the structural changes involved in the XY relaxation in unfolded RNase, except that it does not involve changes in equilibrium conformational features that are urea sensitive. In spite of the lack of a urea effect on the equilibrium distribution of XY species, urea does exert an enormous effect on the relaxation time τ_{XY} [item (VI) (B)]. Its value is increased by nearly 100 times in going from 0.3 to 5 M urea, implying that the activated state lying on the pathway between the equilibrium XY species is seriously destabilized by urea even though the initial and final states are not. The XY process bears many similarities to the CT process, noted above, and conceivably could be due to the isomerization of a peptide bond(s) other than the proline-93 bond. However, because of this large sensitivity of relaxation time to urea concentration, which is not seen for the CT process, and because of the small E_a of 8 kcal/mol, we are inclined not to suggest this interpretation at present.

In the following paper (Lin & Brandts, 1983c), a very simple model is proposed which will account for all of the above experimental characteristics of the RNase unfolding and refolding reactions, including the interesting dependence of refolding amplitudes on the relative values of relaxation times τ_{CT} and τ_{XY} (item VIII).

Registry No. RNase A, 9001-99-4; urea, 57-13-6.

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Mechanism for the Unfolding and Refolding of Ribonuclease A. Simulations Using a Simple Model with No Structural Intermediates†

Lung-Nan Lin and John F. Brandts*

ABSTRACT: A simple mechanism is proposed to account for the folding and unfolding of RNase A in aqueous urea solution. This is based on the existence of three independent structural processes, each of which gives rise to counterpart relaxations in base-line unfolding and base-line refolding experiments. The fast ND process involves the actual conformational changes which take place, while the slower CT and XY processes are due to urea-insensitive chain equilibrations in the denatured state. The CT process is identified as the cis–trans isomerization of proline-93, while the origin of the XY process is not known. If numerical values for the minimal set of parameters are assumed, the model can be used to simulate base-line unfolding and baseline refolding experiments. The relaxation times and amplitudes of the different phases seen in the simulated kinetics agree under all conditions with the corresponding experimental parameters, within the experimental error. This includes values obtained from absorbance, fluorescence, and double-jump experiments in both the unfolding and refolding directions and over a concentration range of urea from 0.3 to 8.0 M. It is concluded that none of the kinetic phases which can be seen directly in stopped-flow or manual-mixing experiments are due to populating structural intermediates along the folding pathway. There is, however, evidence from amplitude measurements that there is a urea-sensitive process which occurs too fast to be seen directly by stopped-flow methods. It is suggested that this is due to a fast equilibrium between the urea-denatured form, closely akin to a random chain, and the water-denatured form, which has less-extended dimensions and possibly some local ordered structure.

There is an extremely large amount of data in the literature on the unfolding and refolding of RNase A, much more so than for any other protein. It is in many ways the archetypical globular protein for the testing of mechanisms for folding. Most of the thermodynamic evidence (Lumry et al., 1966; Brandts & Hunt, 1967; Tanford, 1968; Jackson, 1970; Privalov & Khechinashvili, 1974; Brandts et al., 1975; Y. Saito and A. Wada, unpublished experiments) is quantitatively consistent with the idea that the equilibrium unfolding and refolding closely approximates a two-state process with no detectable concentration of true structural intermediates. On the other

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hand, base-line refolding kinetic experiments show obvious complexities both for RNase A (Nall et al., 1978; Cook et al., 1979; Schmid, 1981; Schmid & Blaschek, 1981) and for RNase S (Labhardt & Baldwin, 1979a,b), and these have frequently been attributed to the existence of stable structural intermediates on the refolding pathway under strongly native conditions.

In this paper, we seek to find the simplest model for the folding and unfolding of RNase which is quantitatively consistent with known thermodynamic and kinetic data. Intermediate states will not be included in the model since they are unnecessary in terms of fitting the existing experimental data. Thus, the model presented here is very different from others that have been proposed for RNase. Although we will rely heavily on recent kinetic data obtained in our laboratory (Lin & Brandts, 1983b,c) using urea as the denaturant, these data are very similar to results from many other laboratories which used Gdn·HCl as the denaturant.

Methods

Kinetic simulations were carried out for the model depicted in eq 2 by using a computer. For base-line refolding simulations it was assumed that k_{DN} is very large and that k_{ND} is small compared to k_{DN}. Initial fractional concentrations for D_{CX}, D_{CY}, D_{TX}, and D_{TY} were assumed to be 0.20, 0.53, 0.074, and 0.196, respectively. Concentrations of the individual molecular species during refolding were obtained by summing the concentration changes which occur over successive small time intervals Δt, these changes being given by the equations

$$\Delta(D_{CX}) = [(D_{CY})k_{YX} + (D_{TX})k_{TC}]\Delta t$$

$$\Delta(D_{TX}) = [-(D_{TX})(k_{TC} + k_{XY}) + (D_{TY})k_{YX}]\Delta t$$

$$\Delta(D_{CY}) = [-(D_{CY})(k_{YX} + k_{CT}) + (D_{TY})k_{TC}]\Delta t$$

$$\Delta(D_{TY}) = [-(D_{TY})(k_{TC} + k_{YX}) + (D_{CY})k_{CT} + (D_{TX})k_{XY}]\Delta t$$

where parentheses refer to fractional concentrations. The values for the rate constants used in these equations were determined as described under Results. Since the kinetics become slower at higher urea concentrations, the time interval Δt was increased from a value of 0.05 s at 0.31 M urea to 0.5 s at 5.2 M urea.

For base-line unfolding simulations, it was assumed that k_{ND} is very large and that k_{DN} is small compared to k_{ND}. Initial fractional concentrations were assumed to be 1.0 for D_{CX} and zero for all other species. Concentrations of individual molecular species during unfolding were again obtained by summing the concentration changes which occur over successive small time intervals Δt, which was taken as 0.3 s at 4.7 M urea and 0.5 s at 8 M urea. The following equations were used to calculate concentration changes:

$$\Delta(D_{CX}) = [-(D_{CX})(k_{CT} + k_{XY}) + (D_{TX})k_{TC} + (D_{CY})k_{YX}]\Delta t$$

$$\Delta(D_{CY}) = [-(D_{CY})(k_{YX} + k_{CT}) + (D_{TY})k_{TC} + (D_{CX})k_{XY}]\Delta t$$

$$\Delta(D_{TX}) = [-(D_{TX})(k_{TC} + k_{XY}) + (D_{TY})k_{YX} + (D_{CX})k_{CT}]\Delta t$$

$$\Delta(D_{TY}) = [-(D_{TY})(k_{TC} + k_{YX}) + (D_{CY})k_{CT} + (D_{TX})k_{XY}]\Delta t$$

Once the time dependence of the concentration of each molecular species is known, either absorbance or fluorescence decay curves could be easily calculated by assigning intrinsic absorbance or fluoresence values to each molecular species. For absorbance, a value of 0 was assigned to N_{CX} and a value of 1.0 was assigned to each of the four denatured species. For fluorescence, a value of 0 was assigned to N_{CX}, a value of 1.0 assigned to D_{CX} and D_{CY}, and a value of 2.5 was assigned to D_{TX} and D_{TY}. Individual relaxation times and amplitudes were obtained by plotting the composite decay curves semilogarithmically and then peeling off exponentials.

Results

The Model. The first three papers in this series (Lin & Brandts, 1983a–c) described experimental studies of the folding and unfolding of RNase. Briefly summarized, the following was found. (1) A total of six first-order relaxations have been identified, three in the folding direction and three in the unfolding direction. These six relaxations were shown to be due to three molecular processes, which are driven in different directions during base-line folding and unfolding. (2) The fast ND process is the only one involving extensive conformational changes. (3) Both the XY and CT slow processes occur in the unfolded molecule. They produce no intrinsic change in absorbance when they occur and are therefore only visible in the refolding direction since they couple to the ND process by mass action effects. The CT process does produce an intrinsic change in fluorescence, due to a local chain effect, and is therefore seen directly in the unfolding direction as well. (4) Although the relaxation times depend on final urea concentration, the equilibrium distribution of species is urea insensitive for both the XY and CT processes. (5) The CT process is due to the isomerization of proline-93, which is 100% cis in native RNase and 70% cis in unfolded RNase.

The three molecular processes can be represented as

$$\begin{aligned} &N \underset{k_{DN}}{\overset{k_{ND}}{\rightleftharpoons}} D && \text{(fast conformational change)} \\ &C \underset{k_{TC}}{\overset{k_{CT}}{\rightleftharpoons}} T && \text{(slow chain equilibration)} \\ &X \underset{k_{YX}}{\overset{k_{XY}}{\rightleftharpoons}} Y && \text{(slow chain equilibration)} \end{aligned} \qquad (1)$$

If it is assumed that these three processes occur completely independently of each other, then the appropriate mechanism will be

$$\begin{array}{ccccc} N_{CX} & \underset{k_{DN}}{\overset{k_{ND}}{\rightleftharpoons}} & D_{CX} & \underset{k_{TC}}{\overset{k_{CT}}{\rightleftharpoons}} & D_{TX} \\ & & (0.20) & & (0.074) \\ & & k_{YX} \uparrow \downarrow k_{XY} & & k_{XY} \downarrow \uparrow k_{YX} \\ & & D_{CY} & \underset{k_{TC}}{\overset{k_{CT}}{\rightleftharpoons}} & D_{TY} \\ & & (0.53) & & (0.196) \end{array} \qquad (2)$$

where slow isomerization C ⇌ T occurs horizontally and the slow X ⇌ Y process of unknown origin occurs vertically. The numbers in parentheses below each of the four denatured species correspond to equilibrium fractions and will be discussed shortly. It has also been assumed in arriving at this mechanism that only the CX form of the denatured protein can refold into the native state; i.e., the species N_{TX}, N_{TY}, and N_{CY} are highly unstable. This is in agreement with all of our experimental data, as discussed at length for the CT process (Lin & Brandts, 1983b,c) and as will be seen in these simulations.

The assumption of independent processes means that

$$(D_{CX})/(D_{TX}) = (D_{CY})/(D_{TY}) = k_{TC}/k_{CT} \qquad (3)$$

$$(D_{CX})/(D_{CY}) = (D_{TX})/(D_{TY}) = k_{YX}/k_{XY} \qquad (4)$$

where the parentheses signify equilibrium fractions for the four denatured species. Furthermore, the requirement for constant mass means that

$$(N_{CX}) + (D_{CX}) + (D_{CY}) + (D_{TX}) + (D_{TY}) = 1 \quad (5)$$

The four-cornered mechanism of eq 2 is exactly the same as one suggested earlier (Brandts et al., 1975) for a protein which has two isomerizing prolines, both of which must be in the "correct" configuration before refolding can occur. In the present case, however, only the CT process has been shown to be due to proline isomerization. Although the XY process seems to be due to a chain equilibration of some type, it cannot yet be identified as proline isomerization (Lin & Brandts, 1983c).

Input Data. Numerical values will be assigned to the minimum number of parameters which are necessary so that the behavior of the above model is determined. These are as follows.

(I) Urea-Independent Parameters. (A) An equilibrium concentration of 27% trans for proline-93 in unfolded RNase was assigned. This agrees closely with the value of 30% trans determined directly by proteolysis (Lin & Brandts, 1983b). With this assignment, then

$$k_{TC}/k_{CT} = 0.73/0.27 = 2.70 \quad (6)$$

(B) An equilibrium concentration of 20% was assigned for the fast-refolding denatured species

$$(D_{CX}) = 0.20 \quad (7)$$

(C) Relative values for intrinsic absorbance and fluorescence were assigned. (1) For absorbance, a relative value of 0 for the native N_{CX} species and a value of 1.0 for all denatured species were used. (2) For fluorescence, a relative value of 0 for the native N_{CX} species, a value of 1.0 for the two denatured species D_{CX} and D_{CY} which have proline-93 in the cis form, and a value of 2.5 for the two denatured species D_{TX} and D_{TY} which have proline-93 in the trans form were used.

(II) Urea-Dependent Parameters (M = Molarity). (A) One rate constant was assigned for the X $\rightleftharpoons$ Y process

$$k_{YX}^{-1} = 15 + 30(M)^{2.3}\ s \quad (8)$$

(B) One rate constant was assigned for the C $\rightleftharpoons$ T process

$$k_{TC}^{-1} = 80 + 40(M)^{0.7}\ s \quad (9)$$

No mechanistic implications are intended from the form of eq 8 and 9. These are simply convenient analytical expressions for reproducing the relaxation times over the range of urea concentrations which are of interest here. It is unlikely that these expressions will be quantitatively correct at urea concentrations higher than those used in our experimental study (Lin & Brandts, 1983c), which were limited to 4.8 M for the XY phase and 8.0 M for the CT phase.

Simulation of Base-Line Refolding Curves. From the intrinsic characteristics of the simple model together with the assigned values of the minimal parameters, relaxation curves can now be determined by computer simulations. Throughout these calculations it will be assumed that the conformational ND process is "very fast" and no attempt will be made to reproduce actual relaxation times for this fast phase (see Methods).

Before examining relaxation curves, it is instructive to look at the equilibrium concentrations of denatured species. In addition to the assigned value of 0.20 for the fast-refolding species D_{CX}, the values for D_{CY}, D_{TY}, and D_{TX} can now be easily determined from eq 3–7 and are 0.53, 0.196, and 0.074, respectively. These values have been included in parentheses

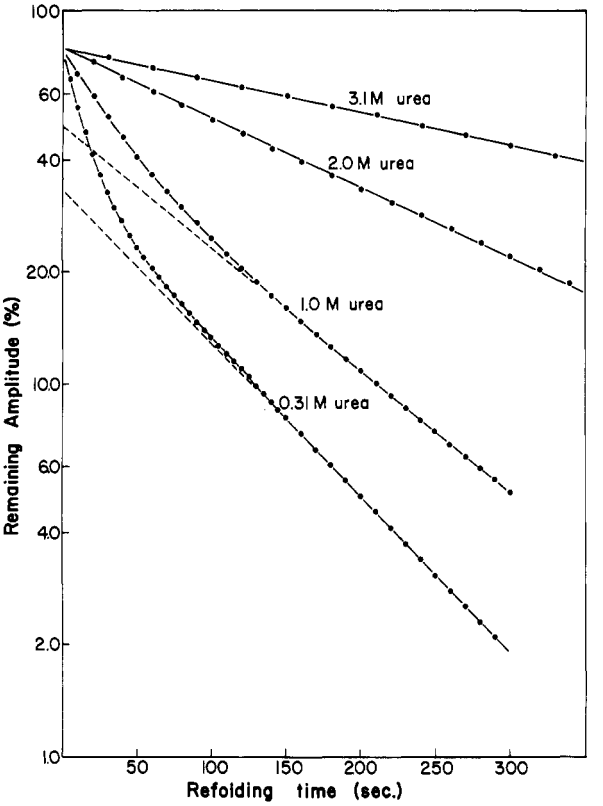


FIGURE 1: Simulations of refolding kinetics of RNase at various final urea concentrations, according to the model of eq 2. At zero time, the fractional equilibrium concentrations of D_{CX}, D_{CY}, D_{TX}, and D_{TY} are as indicated in eq 2. The rate constants at various final urea concentrations are calculated from eq 8 and 9 and from the above equilibrium concentrations. Points represent the simulated data. The detailed procedures are described under Methods.

in the model of eq 2. The unequal distribution of molecules among the three slow-refolding forms produces some very unusual changes in amplitude as a function of urea concentration. When the velocity of the XY process is much faster than the velocity of the CT process (i.e., at low urea concentration), then the D_{CY} species (53%) will refold with a relaxation time characteristic of the XY process while both the D_{TX} and D_{TY} species (combined population of 27%) will refold with a relaxation time characteristic of the slower CT process. However, when the velocity of the XY process is much slower than that of the CT process (at high urea concentration), then both the D_{CY} and D_{TY} species (combined population of 72.5%) will refold with a relaxation time characteristic of the XY process, while only the unstable D_{TX} species (7.5%) will refold with a relaxation time characteristic of the faster CT process. Because the amplitudes of the two phases differ by almost a factor of 10, the decay curves will appear to conform to a single-exponential function over a wide range of intermediate urea concentrations before the small CT phase is actually sufficiently faster than the XY phase to make it experimentally distinguishable. It will be recalled that these properties of the model coincide almost exactly with the experimentally observed amplitudes (Lin & Brandts, 1983c) as a function of urea concentration. The key to this curious amplitude behavior is, of course, the D_{TY} species which must undergo *both* the Y $\rightarrow$ X and the T $\rightarrow$ C process before it can refold. Thus, the amplitude (19.6%) for refolding of this species will appear in either the XY or CT relaxation, depending on which is rate limiting.

Some computer-simulated absorbance curves for base-line refolding, according to the model of eq 2, are shown in Figure

Table 1: Comparisons of Simulated Base-Line Refolding Kinetic Data with Experimental Kinetic Data

final urea conen (M)		XY phase[a] amplitude	XY phase[a] τ (s)	CT phase[a] amplitude	CT phase[a] τ (s)
0.31	exptl	0.50	22	0.30	85
	calcd	0.47	17	0.33	105
1.0	exptl	0.16	38	0.64	120
	calcd	0.30	35	0.50	135
2.0	exptl	0.80	280	0	
	calcd	0.80	235	0	
3.1	exptl	0.80	510	0	
	calcd	0.80	505	0	
4.1	exptl	0.74	780	0.06	190
	calcd	0.76	900	0.04	190
5.2	exptl	0.73	1500	0.07	220
	calcd	0.75	1500	0.05	200

Limiting Amplitude[b] ($\tau_{XY} \ll \tau_{CT}$)		f_{ND}	f_{XY}	f_{CT}
absorbance	exptl	0.20	0.53	0.27
	calcd	0.20	0.53	0.27
fluorescence	exptl	0.15	0.38	0.47
	calcd	0.14	0.38	0.48

[a] All experimental data are obtained from absorbance measurements, taken from Table I of the preceding paper (Lin & Brandts, 1983c). [b] Experimental limiting amplitudes shown here are those of final urea concentration of 0.31 M with 0.56 M $(NH_4)_2SO_4$. See Table I of the preceding paper.

1 for final urea concentrations of 0.31, 1.0, 2.0, and 3.1 M urea. The fast ND phase is seen only as an initial zero-time displacement of amplitude from 100 to 80%. The simulated kinetics for the slow phase are seen to bear a striking resemblance to the corresponding experimental curves at the same urea concentrations, plotted in Figure 1 of the preceding paper (Lin & Brandts, 1983c). Two separate slow relaxation processes are clearly evident for refolding into 0.31 and 1.0 M urea while the curves at 2.0 and 3.0 M urea correspond to single-exponential decay within very small deviations. If these simulated curves are treated in exactly the same way as were the experimental data, by peeling off exponentials, then the resulting τ_{XY} and τ_{CT} relaxation times and amplitudes are virtually identical with the corresponding experimental values, within experimental errors.[1] The comparisons between experimental and simulated data are shown in Table I. For example, at 0.31 M urea the τ_{CT} phase has an experimental amplitude of 30% and a relaxation time of 85 s while the corresponding values from the simulated curve are 33% and 105 s. For the τ_{XY} phase at 0.31 M, the experimental parameters are 50% and 22 s while the simulated values are 47% and 17 s. From the calculated concentrations in the simulation, it was ascertained that the τ_{XY} phase is due almost exclusively to the refolding of the D_{CY} species while the τ_{CT} phase results from the refolding of both the D_{TY} and D_{TX} species.

[1] The method of peeling off exponentials, used for analysis of the simulated curves, is an arbitrary procedure and will lead to a smaller number of exponential phases than would be obtained from the exact mathematical solution of the mechanism of eq 2. For example, two exponential phases with nearly the same relaxation time will be treated as a single phase when exponentials are peeled off. Phases of very low amplitude will always be overlooked unless their relaxation time differs substantially from the relaxation times of the phases of larger amplitude. This presents no serious problems in the present instance. Within very small tolerances, all of our simulated curves can be fit with no more than two exponential functions under all conditions which were examined. Furthermore, by examining concentrations of individual denatured species in the simulations, it can be seen that the decay of each molecular species occurs with a relaxation time which is very close to one of the relaxation times obtained by peeling off exponentials.

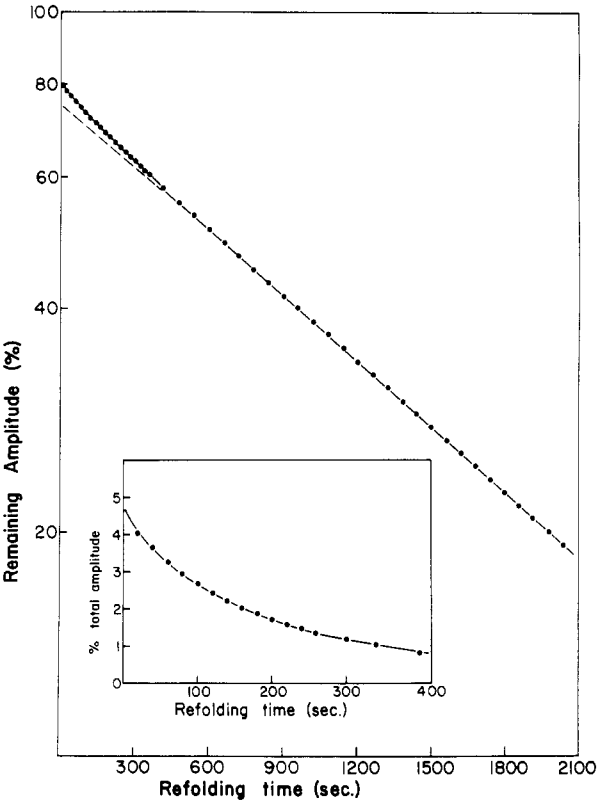


FIGURE 2: Simulations of refolding kinetics of RNase at a final urea concentration of 5.2 M. The procedures are the same as those of Figure 1. The inset shows the small faster phase which occurs during the first 400 s, obtained after peeling off the slower phase.

For the single-exponential decay curves at 2.0 and 3.1 M urea in Figure 1, relaxation times τ_{XY} are 235 and 505 s, respectively. The corresponding experimental relaxation times are 280 and 510 s, respectively. Careful examination shows some deviations from single-exponential behavior in the simulated curves at 2.0 and 3.1 M urea, but these are much too small to have been detected in the experimental data.

The simulated curves at higher urea concentrations of 4.1 M (Table I) and 5.2 M (Table I and Figure 2) again show two phases, although of very unequal amplitudes. The smaller and faster of these has an amplitude of 5% and a relaxation time τ_{CT} of 200 s at 5.2 M, compared with experimental values of 7% and 220 s. This phase results primarily from the refolding of the species D_{TX}. The larger slow phase has a simulated amplitude of 75% and relaxation time of 1500 s, compared to the experimental values at 5.2 M urea of 73% and 1500 s. Agreement between actual and predicted behavior at 4.1 M is equally good (Table I).

Its ability to quantitatively simulate the characteristics of this small phase, seen only during refolding at high urea, certainly adds much credibility to the simple model which we propose. In fact, this phase had not been seen experimentally until after the results of the simulations were available to us. The prediction of the small phase in the simulations prompted us to do refolding experiments at urea concentrations higher than those previously used, whereupon it was observed experimentally.

In general, relaxation times τ_{XY} and τ_{CT} obtained from simulated fluorescence refolding are virtually identical with those obtained from simulated absorbance refolding under the same conditions, in agreement with the experimental situation, and are therefore not shown in Table I. However, fluorescence amplitudes for the individual phases are quite different from absorbance amplitudes since the relative amplitude of the τ_{CT}

Table II: Comparisons of Simulated Base-Line Unfolding Kinetic Data with Experimental Kinetic Data

method	final urea concn (M)		XY phase[a] amplitude	XY phase[a] τ (s)	CT phase[a] amplitude	CT phase[a] τ (s)
fluorescence	4.7	exptl	0		0.30	155
		calcd	0		0.29	145
	8.0	exptl	0		0.30	160
		calcd	0		0.29	190
double jump[b]	4.7	exptl	0.50	300	0.30	170
		calcd	0.50	300	0.30	145
	8.0	exptl			0.30	180
		calcd			0.30	190
absorbance	4.7	exptl	no slow phase amplitude			
		calcd	no slow phase amplitude			
	8.0	exptl	no slow phase amplitude			
		calcd	no slow phase amplitude			

[a] All experimental data are taken from Table II of the preceding paper. [b] For double-jump experiments the relaxation times are for unfolding, but the amplitudes are those observed for refolding after the jump back to the native-state buffer.

phase is considerably enhanced in fluorescence, compared to absorbance, due to the intrinsic fluorescent properties of those denatured molecules having proline-93 in the trans form (i.e., D_{TX} and D_{TY}). A comparison of limiting amplitudes, when $\tau_{XY} \ll \tau_{CT}$, for both fluorescence and absorbance detection is shown in Table I. The comparison of experimental and simulated amplitudes shows that the simple model of eq 2 is able to quantitatively predict refolding amplitudes for all three phases in both absorbance and fluorescence. The agreement is in fact better than the expected experimental errors.

Simulation of Base-Line Unfolding Curves. Base-line unfolding experiments can also be simulated for the model of eq 2. Since exactly the same states are assumed to be present in the unfolding direction as in the refolding direction and since the same input parameters will be used, the agreement of the unfolding simulations with experiment is really a test of whether the relaxations seen during unfolding are in fact due to the counterpart processes of those seen during refolding.

The model predicts (Table II) that no slow phase will be seen by absorbance monitoring, in agreement with experiment. In addition to the fast ND phase, a slow CT phase will be seen in fluorescence resulting from populating the D_{TX} and D_{TY} states. The data in Figure 3 show a calculated fluorescence unfolding curve at 4.7 M urea, compared to the actual measured curve obtained under the same conditions. The agreement between simulations and experiment is equally good for fluorescence unfolding in 8.0 M urea (Table II).

Simulations of double-jump experiments have also been carried out, with the results listed in Table II, for unfolding into 4.7 and 8.0 M urea. Not only is there excellent agreement with experiment for the τ_{CT}' phase at both concentrations, but the predicted amplitude and relaxation time for the τ_{XY}' phase at 4.7 M urea are also very good. Since this phase is silent in both absorbance and fluorescence, it can only be "seen" by double-jump methods. The relaxation time is about twice as large for the XY process as for the CT process at 4.7 M urea.

Discussion

The model of eq 2 is able to simulate all of our experimental data on the base-line unfolding and refolding of RNase in urea. The agreement with experimental data is approximately the same as the expected experimental error, on average. Within these small differences, the model accounts for the numerical values of all relaxation times and all relative amplitudes for the XY and CT processes in the refolding direction from 0.3 to 5 M urea and in the unfolding direction from 4.7 to 8 M urea, even though only one urea-dependent input parameter is assigned for each of the two slow processes. It offers a simple explanation for the fact that relaxation times measured by absorbance and fluorescence are the same, whereas the relative amplitudes are quite different. Furthermore, it explains why certain phases seen in the refolding direction are not visible in the unfolding direction, even though double-jump experiments show that they are actually taking place.

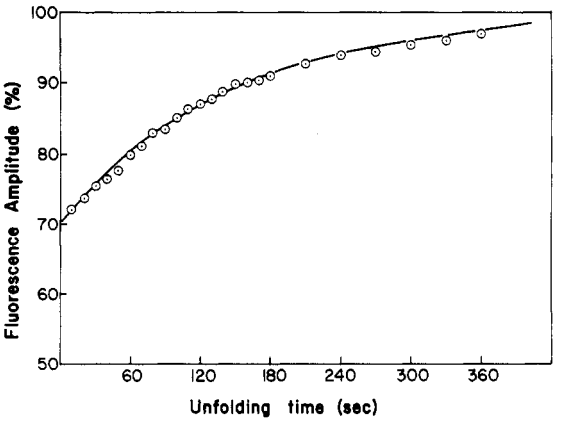


FIGURE 3: Comparisons of the simulated unfolding kinetics of RNase with that obtained directly from fluorescence experimental data at a final urea concentration of 4.7 M. The model of eq 2 was used for simulation. At zero time, (D_{CX}) was taken as 1.0 while all other concentrations were zero. The rate constants for simulation were obtained as in Figure 1. The solid line represents the simulated data, while the points represent the experimental fluorescence data obtained from studies described in the preceding paper (Lin & Brandts, 1983c).

The ability of the model to predict the highly unusual amplitude changes seen in the experimental refolding curves deserves special mention, since it seems extremely unlikely that this would happen if the chosen model were incorrect. The experimental amplitude for the CT phase is ca. 30% at low urea where the relaxation time for the CT phase is much slower than for the XY phase, while it decreases more than 4-fold to ca. 7% at higher urea concentrations when the relaxation time for the CT phase is much faster than for the XY phase. At intermediate urea concentrations, only a single phase is seen experimentally. In terms of our model, these amplitude changes occur because the molecular species D_{TY} must undergo *both* the T → C and the Y → X changes before it can refold. The amplitude associated with refolding of the D_{TY} species (i.e., 19.6%) will then occur in the slower of the two phases, which means it will be in the CT phase refolding into low urea but will be in the XY phase for refolding into high urea. Thus, the very unusual dependence of the slow phase amplitudes on urea concentration is easily accommodated by the model, since this is a necessary property of any four-cornered model based on the occurrence of two independent slow processes in the unfolded state. That is, the slow refolding species which is diagonally opposite the fast refolding species in the four-cornered scheme (cf. eq 2) will always refold in the slower of the two slow phases.

The overall success of the model provides strong evidence that the molecular species which are populated during base-line refolding into low urea concentrations are the same species which are populated during base-line unfolding into high urea concentrations, since this is the primary assumption which has been used in the model of eq 2. Thus, unfolding data for RNase can be quantitatively predicted from refolding data with no further assumptions, as we have shown. This conclusion is contrary to previous suggestions that additional well-folded states become populated only during base-line

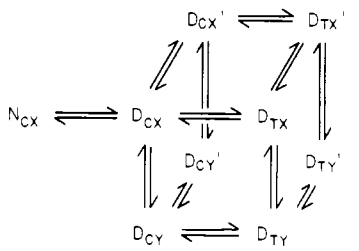
refolding for both RNase A (Nall et al., 1978; Cook et al., 1979; Schmid, 1981; Schmid & Blaschek, 1981) and RNase S (Labhardt & Baldwin, 1979a,b) in Gdn·HCl. In fact, these results show that, at the present level of experimental detection, the complete set of kinetic data for both unfolding and refolding can be nicely fit by a mechanism which assumes that no true structural intermediates are populated as the reaction progresses in *either* direction. We are not proposing that structural intermediates do not exist, only that they are undetectable in these experimental kinetic data. Surely they do exist, but the concentration levels at which they will be populated is a matter of great uncertainty. At any rate, the obvious complexities in the refolding kinetics appear to be due to two subtle chain equilibrations in unfolded RNase and not to structural intermediates. Since our data obtained on RNase A in urea solutions bear unmistakable similarities to kinetic data obtained on both RNase A and RNase S in Gdn·HCl, we feel that many features of these systems might also be equally well explained by the same model.

Needless to say, the proposed mechanism is also in good agreement with many thermodynamic studies, which show that RNase denaturation conforms, within small tolerances, to a two-state unfolding model.

Even though all of the kinetic and thermodynamic data discussed thus far can be readily accounted for by neglecting structural intermediates and assuming the existence of only a single native state and a single denatured state [i.e., one macroscopic state (Lumry et al., 1966) consisting of four slowly equilibrating species of unfolded RNase], the real situation is probably not this simple. It has been known for some time that denatured proteins in water do not exist as complete random coils, whereas they are probably very close to random coils in 10 M urea or 6 M Gdn·HCl [reviewed by Tanford (1968)]. For example, significant differences are found in the CD, NMR, fluorescence, and absorption spectra when denatured proteins in water are compared to denatured proteins in aqueous urea or aqueous Gdn·HCl solutions. Intrinsic viscosities suggest that the coil dimensions of urea- or Gdn·HCl-denatured proteins could be twice as large as are the dimensions in water. Also, Baldwin and his colleagues (Schmid & Baldwin, 1979; Kim & Baldwin, 1980) have shown that denatured RNase in water has some slowly exchanging protons which are not seen for denatured RNase in high Gdn·HCl, presumably reflecting more complete solvation of the polypeptide chain in the presence of the strong denaturant.

Although it seem certain then that some "structure" is present in denatured proteins in water, the precise nature of this structure is unknown. It has been suggested from CD measurements (Labhardt, 1982a,b) that thermally denatured RNase has lost all of its β sheet but that a small fraction of the residues (ca. 10%) remain in a helical configuration which is lost completely only after the addition of 9 M urea or 6 M Gdn·HCl. The very gradual CD change as a function of the amount of denaturant added shows that, whatever the nature of the structure which remains, it is not highly cooperative. This lack of cooperativity suggest to us that the kinetics for formation and loss of this denaturant-sensitive structure will be very fast, in analogy with the known relaxation times for helix–coil transitions of homopolypeptides which also possess low cooperativity because of their one dimensionality (Brandts, 1969).

Incorporating the above ideas, an expanded eight-cornered form of the model in eq 2 can be illustrated as



(10)

where the four primed species D_{CX}', D_{CY}', D_{TX}', and D_{TY}' are the more-unfolded denatured forms stable in high urea and the corresponding unprimed species are the less-unfolded forms stable in water. The transitions $D \rightleftharpoons D'$ are illustrated as two-state transitions in eq 10 for convenience, but they will probably be much less cooperative than this, as already suggested.

Our experimental data show that the relative amplitudes of all three refolding phases are unchanged as the equilibria in eq 10 are shifted from the D to the D′ forms, by increasing the initial concentration of urea from 3 to 10 M, so that the *ratio* of equilibrium concentrations for any two primed denatured species must be the same as the *ratio* for the same two unprimed species (i.e., $D_{CY}'/D_{CX}' = D_{CY}/D_{CX}$, $D_{CX}'/D_{TX}' = D_{CX}/D_{TX}$, etc.).

If the kinetics for the transition from any primed species to the corresponding (i.e., same subscripts for XY and CT) unprimed species are too fast to resolve by stopped-flow techniques, as implied above, then we would not expect to find any relaxation processes in our data *that are rate limited* by these $D \rightleftharpoons D'$ transitions, and we do not. However, evidence showing that these transitions do take place can be obtained by comparing stopped-flow absorbance amplitudes of complementary unfolding and refolding experiments, which are carried out between the same two sets of conditions, which shall be referred to as S_1 and S_2. The former refers to zero or low urea and the latter to high urea concentration. Consider the two processes

base-line unfolding: native RNase (S_1) → denatured RNase (S_2)

base-line refolding: denatured RNase (S_2) → native RNase (S_1)

where the absolute values of the experimental absorbance amplitudes, *for all relaxations seen in stopped-flow experiments*, will be denoted as $|\Delta A_U|$ and $|\Delta A_R|$, respectively. In the refolding experiment, the $D' \rightarrow D$ transitions will occur as the first step and the amplitude will be lost in the dead time of the instrument. However, in the unfolding experiment, the amplitude of the $D \rightarrow D'$ transition will be seen since this process is the last visible process to occur and will therefore be rate limited by the slower, more cooperative $N \rightarrow D$ process according to the mechanism of eq 10. Therefore, if the $D \rightleftharpoons D'$ transitions do occur very fast and give rise to intrinsic changes in absorbance, we would expect to find that

$$|\Delta A_U|/|\Delta A_R| > 1.0$$

This effect can be easily observed experimentally. For example, the above ratio is found to be ca. 1.20 when the S_1 conditions are 1 M urea, pH 3.5, and the S_2 conditions are 5 M urea, pH 3.4, both at 25 °C. This leads to a minimum estimate[2] of 20% for the fraction of the total amplitude change

which occurs in the D ⇌ D′ transitions when the urea concentration is changed from 5 to 1 M. This excess amplitude is substantially smaller if the complementary unfolding and refolding experiments are carried out by changing the pH rather than the urea concentration. For S_1 of 4 M urea, pH 6, and S_2 of 4 M urea, pH 3, the corresponding amplitude ratio is 1.04. Thus, the equilibrium for the D ⇌ D′ transitions is much less sensitive to pH changes than to changes in urea concentration.

The large observed effect of urea on the relaxation time for the XY process can also be rationalized nicely in terms of the expanded model of eq 10. For example, the process occurring in low final urea concentration

$$D_{CX} \underset{k_{YX}}{\overset{k_{XY}}{\rightleftharpoons}} D_{CY}$$

can be compared to the same process occurring in high final urea concentration

$$D_{CX}' \underset{k_{YX}'}{\overset{k_{XY}'}{\rightleftharpoons}} D_{CY}'$$

where, as discussed above, it is known that the *ratio* of rate constants k_{XY}/k_{YX} must be the same as the ratio k_{XY}'/k_{YX}'. The same would be true for the identical XY process involving the trans form of proline-93 rather than the cis form. Urea was shown experimentally to have a large effect in slowing down the XY process, so that the individual rate constants k_{XY}' and k_{YX}' in high urea must be considerably smaller than k_{XY} and k_{YX}, even though the ratios are the same. It is quite common to find that additives alter the kinetics of a reaction without having any detectable effect on the thermodynamics, and this is apparently another instance where this occurs. We would conclude then that it becomes much more difficult kinetically for the XY process to occur as denatured RNase assumes larger hydrodynamic dimensions and becomes closer akin to a random chain. This is consistent with the idea that the XY process could involve passing through an activated complex with relatively low chain entropy, which thereby becomes destabilized when the equilibrium X and Y structures gain in chain entropy by the addition of urea or Gdn·HCl. One process which seems to fit these criteria has already been suggested for RNase by Nall et al. (1978), who showed that in the unfolded polypeptide chain the disulfide-bonded loops can exist in two isomeric states, only one of which is "correct" for refolding to the native protein. Interconversion of these two isomers must occur by a low-entropy path which requires pulling one loop, or one end of the polypeptide chain, through another loop, according to Nall et al. This process would be expected to be considerably slower in high concentrations of urea or Gdn·HCl, where the larger equilibrium chain dimensions will make it more costly entropically to carry out the loop-threading process.

Although the above idea is attractive, there are other processes which could also be responsible for the XY relaxation. It was shown that the rate of isomerization of proline-93 depends not only on local sequence effects but also to some extent on overall chain dynamics (Lin & Brandts, 1983b,c). No slow relaxation has as yet been identified for the isomerization of proline-114, which is also cis in native RNase. This occurs in a region of the chain which is very close to another proline (proline-117) and not far from a disulfide loop (cysteine-110), and it is at least possible that isomerization kinetics for this proline could be even more sensitive to chain dynamics, and hence to the presence of urea or Gdn·HCl, than is proline-93. It has been suggested that no relaxation will be seen for proline-114 because it is "nonessential" and can be accommodated in folded RNase in either the cis or trans form (Kim & Baldwin, 1982). However, there is as yet no experimental evidence showing that this is true, so its possible involvement in the XY process cannot be disregarded until further evidence is available.

In summary, our results show that all of the relaxations of RNase which are slow enough to be seen directly in stopped-flow measurements can be accounted for in terms of a fast conformational step and two slow chain equilibrations over the range of conditions examined. The simple four-cornered model which quantitatively simulates RNase unfolding and refolding is identical with one proposed earlier (Brandts et al., 1975) and involves no structural intermediates. However, amplitude measurements from complementary refolding and unfolding experiments show that there is another very fast conformational process outside the stopped-flow time range. It is the first step in the refolding direction and the last absorbance-visible step in the unfolding direction. This very fast process is due to the interconversion between water-denatured and urea-denatured RNase.

Of the four processes identified for RNase, the two involving conformational changes are fast and give rise to large changes in intrinsic absorbance, and the two involving chain equilibrations are slow and do not produce any change in intrinsic absorbance. We would suggest that this pattern may also hold for other small proteins. If so, then useful information on true structural intermediates involved in the folding pathway will only come from studying the fast phases under conditions where these are uncoupled from the slower chain equilibrations. Up to the present time, almost all detailed kinetic data on protein folding is on the slow phases.

One final cautionary comment should be made on restrictions in the use of the model of eq 2. This model has been derived solely on the basis of data obtained under base-line refolding and base-line unfolding conditions, and the only molecular species included in the mechanism are those which are directly responsible for relaxations that occur slower than the dead time of the stopped-flow apparatus. Because of the base-line conditions, equilibria associated with faster processes than can be resolved in stopped-flow experiments will make only minor contributions to our base-line data. However, for kinetic data obtained following jumps into the transition region, equilibria due to very fast processes can have substantial effects on the amplitudes and relaxation times of the slower phases due to coupling by mass-action effects. If such fast processes exist for RNase, which seems likely to us, then the amplitudes and relaxation times predicted by the model of eq 2 will not accurately reproduce experimental data near the transition region, since the model will be missing some of the important equilibrium states.

Registry No. RNase A, 9001-99-4; urea, 57-13-6.

[2] This estimate of 20% is probably too small since it does not include corrections for the solvation effect of urea on tyrosyl chromophores. This perturbation can be expected (Donovan, 1965) to increase the molar absorbance by ca. 100 for each tyrosyl chromophore when the change in urea concentration is 4 M in going between S_1 and S_2. If it is assumed that three tyrosines are perturbed in native RNase and six are perturbed in denatured RNase, then the *total* change in absorbance for refolding at 1 M urea will be ca. 15% larger in magnitude than for unfolding in 5 M urea, if identical inverse changes in structure were to occur in the two processes. When this factor is taken into account, then our experimental estimate for the fraction of the total amplitude involved in the D ⇌ D′ transition almost doubles from 20% to 38%.

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Effect of Anions, Chaotropes, and Phenol on the Attachment of Flavin Adenine Dinucleotide to Phenol Hydroxylase†

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ABSTRACT: Monovalent anions, ethylene glycol, or Mg^{2+} inhibits the activity of phenol hydroxylase to a degree essentially compatible with the relative degree of their chaotropic power. All these agents affect the spectrum of phenol hydroxylase in a way indicating changes in the hydrophobic interaction between FAD and the enzyme. All agents, except fluoride, abolish the characteristic shoulders on either side of the maximum at 443 nm, bringing the spectrum of phenol hydroxylase closer to that of free FAD. The effect of fluoride is opposite; the shoulders become more accentuated, indicating a more hydrophobic interaction between FAD and the protein than that in the native enzyme. This interpretation is supported by the results of fluorometric measurements. The fluorescence of enzyme-bound FAD is about 10-fold smaller than that of free FAD. In the presence of several monovalent anions the fluorescence of the enzyme increases significantly, whereas in the presence of fluoride it decreases, instead. Displacement of FAD, at 0.015–0.030 M monovalent anions, not giving easily perceptible changes in the primary spectrum, is indicated by difference spectra in the presence of these agents. Absorption spectra of protein eluates from Sephadex G-25 columns, equilibrated with 0.25–1.0 M azide, cyanide, or thiocyanate, indicate complete removal of FAD. The removal of FAD is, to a varying degree, counteracted by low concentrations of phenol. This protective effect of phenol is discussed with view to its known dual function as both effector and substrate of phenol hydroxylase. Spectrophotometric titration of the binding site(s) for phenol reveals one binding site of high affinity ($K_s \simeq 10^{-6}$ M) and additional binding site(s) of much lower affinity ($K_s \simeq 10^{-3}$ M).

Phenol hydroxylase (EC 1.14.13.7)[1] catalyzes the conversion of simple phenols to their *o*-diol derivatives. The enzyme is an FAD-containing monooxygenase with a strict requirement for NADPH. It was originally purified from the soil yeasts *Trichosporon cutaneum* (Neujahr & Gaal, 1973a,b, 1975) and *Candida tropicalis* (Neujahr et al., 1974) after elimination of chloride ions from the purification procedure. Chloride and certain other anions were found to inhibit the purified enzyme (Neujahr & Gaal, 1973a).

The present paper shows that the inhibition reflects displacement of FAD from its proper attachment to the enzyme. This is partly due to chaotropic phenomena. A few equivalents of phenol per enzyme-bound FAD counteracts, to varying degrees, its release. This correlates with various expressions of the dual function of phenol as both effector and substrate of phenol hydroxylase. Some of the results have been reported previously (Neujahr, 1982).

Materials and Methods

Chemicals. All chemicals were reagent-grade commercial preparations whenever available. Most of them were purchased from Sigma Chemical Co. (St. Louis, MO), except inorganic salts and phenol, which came from Merck (Darmstadt, West Germany). Salts of the monovalent anions were of Suprapur quality. Sephadex products were from Pharmacia Fine Chemicals (Uppsala, Sweden). FAD was purified on 1-mm TLC plates in 1-butanol–acetic acid–H_2O (4:3:3) as solvent.

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[1] Abbreviations: phenol hydroxylase, phenol,NADPH:oxygen oxidoreductase (2-hydroxylating) (EC 1.14.13.7); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TLC, thin-layer chromatography.

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