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Hydrogen exchange and the unfolding pathway of ribonuclease A

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Abstract

Recently, when the kinetic unfolding process of ribonuclease A was monitored by hydrogen exchange (T. Kiefhaber and R.L. Baldwin, Proc. Natl. Acad. Sci. USA, 92 (1995) 2657–2661), all peptide hydrogen bonds were found to undergo rapid exchange in a single kinetic step under conditions where unfolding is slow and the intrinsic rate of hydrogen exchange is fast (pH 8.0, 10°C, 4.5 M guanidinium chloride). Comparison with the unfolding rate measured by circular dichroism indicates that hydrogen exchange is caused by the rate-limiting step of unfolding. No evidence was found for partly unfolded intermediates that are formed slowly enough to be observed by EX1 (unfolding-limited) hydrogen exchange. Some peptide NH protons were found to show, in addition to EX1 exchange, faster EX2 exchange that is base-catalyzed. The EX2 exchange is caused by species that equilibrate rapidly with the native protein at the start of the unfolding process. These species might include rapidly formed unfolding intermediates. We show here that any such unfolding intermediates must have large protection factors because the EX2 reactions of ribonuclease A under these unfolding conditions have protection factors ≥ 2500.

Keywords: Proteins; Unfolding; Ribonuclease A; Hydrogen exchange; Protection factor

1. Bill Harrington and hydrogen exchange

In 1958–1959, at the urging of Bill Harrington and John Schellman, one of us (RLB) took an early sabbatical and went to the Carlsberg Laboratory in Copenhagen to work with Professor Kai Linderstrøm-Lang. He was ill that year and died soon after I left, but his spirit and scientific interests animated the laboratory. Like other visitors, I became fascinated with a problem he had opened up, and which was then his major scientific interest: what are the conformational fluctuations in proteins that allow hydrogen exchange to occur?

Later, beginning in 1979, first with Franz Schmid [1] and then Peter Kim [2], I began work on a closely related problem: how might hydrogen exchange [in conjunction with two-dimensional nuclear magnetic resonance (2D NMR)] be used to determine the folding and unfolding pathways of proteins?

At scientific meetings, Bill Harrington and I often discussed hydrogen exchange and the mechanism of protein unfolding. He was deeply interested in this subject and, like myself, his interest began during his stay in the Carlsberg Laboratory. In his later years, Bill was particularly intrigued by the unfolding–re-

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folding behavior of a low-melting segment of the myosin rod [3] and by the possible function of this segment in the mechanism of muscle contraction. Although, as time progresses, interest in older scientific work fades out, sometimes the problems scientists work on, and their ideas about them, live on for a long time. This will surely be true of Bill Harrington's contributions to science. He had a remarkable ability to spot important scientific problems and also to stimulate other workers. In dedicating this paper to Bill, I look back on the many discussions we had and the great benefit I derived from them.

2. EX1 hydrogen exchange used to monitor the unfolding kinetics of ribonuclease A

The plan of the experiments by Kiefhaber and Baldwin [4] was to use hydrogen exchange as an instantaneous probe of hydrogen bond (H-bond) breakage during unfolding. This plan requires finding conditions where ribonuclease A (RNase A) unfolding is slow compared to the rate of hydrogen exchange in solvent-exposed peptide groups and hydrogen exchange occurs by the EX1 mechanism of Hvidt and Nielsen [5], in which exchange is rate-limited by the rate of unfolding.

$$N \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} U \overset{k_{23}}{\rightleftarrows} U^{*} \qquad (1)$$

$$k_{obs} = k_{12} \text{ if } k_{23} \gg k_{21}, k_{12} \qquad (2)$$

In Eq. 1 unfolding converts the native protein (N) to an exchange-susceptible form U with a rate constant k_{12}; exchange occurs rapidly once U is formed and the rate-limiting step in unfolding-exchange is the unfolding step N → U. Exchange follows the EX1 mechanism, which is not base-catalyzed, and the rate of the exchange step k_{23} does not appear in the expression for the observed exchange rate, k_{obs}. The species U may be either a partly unfolded intermediate or the totally unfolded protein, obtained by global unfolding.

The first test of this plan is whether exchange follows the EX1 mechanism, so that exchange during unfolding is not base-catalyzed. Exchange measurements were made during RNase A unfolding at 10°C, pH 8.0 and pH 9.0, 4.5 M guanidinium chloride

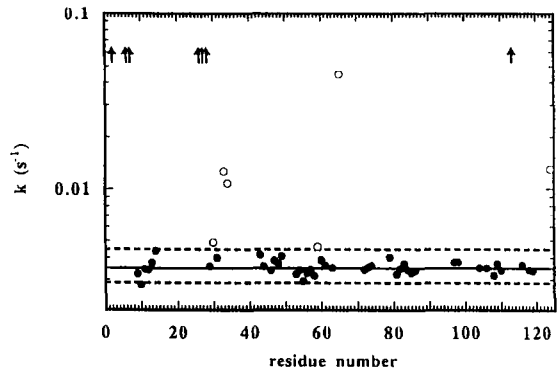


Fig. 1. Hydrogen exchange rates plotted against residue number, when the kinetic unfolding process of RNase A is monitored by hydrogen exchange; unfolding conditions 10°C, pH 8.0, 4.5 M GdmCl (figure reprinted from Kiefhaber and Baldwin [4]). Filled circles show exchange rates that have the same value, independent of residue position, within $\pm 3\sigma$ (σ is the average standard deviation of the individual exchange rate measurements). These protons undergo EX1 exchange by the test that the exchange rates are not base catalyzed (see text). Faster exchanging protons (open circles) undergo both EX1 and EX2 exchange. Arrows denote protons for which exchange is observable but too fast to measure accurately.

(GdmCl), in 90% H_2O, 10% D_2O [4]. The exchange and unfolding processes were started simultaneously by diluting the deuterated native protein in D_2O into a concentrated GdmCl solution in H_2O. At various times, exchange was quenched and the sample was allowed to refold; later, the H_2O solvent was replaced by D_2O and a 2D ^1H NMR spectrum was taken. Exchange was found to proceed slowly, with exchange rates of individual NH protons being comparable to the unfolding rate measured by circular dichroism (CD). The results at pH 8.0 are summarized in Fig. 1. The exchange curves of 49 protons could be measured; 47 have known H-bond acceptor groups. Of the 49 protons, 42 show the same exchange rate within $\pm 3\sigma$ (σ is the average standard deviation of the individual exchange curves). Comparison with the pH 9.0 results shows that the exchange mechanism of the 42 protons at pH 8.0 is not base-catalyzed and therefore is EX1 exchange. The average relaxation time for unfolding-exchange of these protons is 290 s at pH 8.0 and 230 s at pH 9.0. Base-catalyzed exchange would be 10 times faster at pH 9.0 than at pH 8.0, and the observed change in the rate of unfolding-exchange between pH 8.0 and pH 9.0 is much smaller than this, and is consistent

with the rates of unfolding measured at pH 8.0 and pH 9.0 by CD.

The seven protons that show fast unfolding-exchange at pH 8.0 (see Fig. 1) undergo both EX1 and EX2 exchange, as judged by the increase in the rate of unfolding-exchange at pH 9.0. It is likely that their rates of EX1 exchange are the same as the average rate of the other protons which show only EX1 exchange.

Thus, the entire network of peptide H-bonds undergoes rapid H-exchange in a single kinetic step during unfolding. Because all protons show the same EX1 exchange rate (and because its rate is consistent with the unfolding rate measured by CD, see below), this step must be global unfolding. During unfolding-exchange at pH 9.0, eight additional protons show faster exchange than the average, and presumably these protons also undergo both EX1 and EX2 exchange, like the seven faster protons at pH 8.0. Exchange experiments on the native protein in 2.5 M GdmCl, just before the unfolding transition zone (T. Kiefhaber, unpublished results), show that the NH protons which undergo both EX2 and EX1 exchange during unfolding in 4.5 M GdmCl also undergo EX2 exchange in the native protein, while almost all the remaining protons undergo only EX1 exchange.

The second test of the original plan is whether the rate of unfolding measured by EX1 exchange is consistent with the rate of overall unfolding measured by CD. The two unfolding rates are not the same: the relaxation time for unfolding measured by EX1 exchange at pH 8.0 is shorter (290 s) than the relaxation time for CD-detected unfolding (370 s). This effect is expected [4], however, because the exchange process is irreversible whereas CD-detected unfolding is reversible and unfolding under these conditions is slow enough that there is kinetic coupling between unfolding and proline isomerization after unfolding. Simulation of the expected kinetics of EX1 exchange and of CD-monitored unfolding shows that the observed difference between the rates of these two processes is expected [4].

These unfolding-exchange results show clearly that there are no slowly formed, partly unfolded intermediates with open H-bonds that are detectable by EX1 exchange on the overall kinetic pathway of folding. In the following section, we consider whether a different sort of unfolding intermediate, which is formed rapidly and is in equilibrium with the native protein, and therefore is detectable by EX2 exchange, can be observed in unfolding experiments.

3. EX2 exchange of ribonuclease A in unfolding conditions

If U in Eq. 1 (which may refer to a NH proton in either a partly unfolded or wholly unfolded species) equilibrates rapidly with N compared to the rate of the exchange step $U \rightarrow U^*$, then k_{obs} is proportional to the fraction of time the protein spends in the form U and k_{obs} is also proportional to k_{23}, the rate of the exchange step [6].

$$k_{obs} = \left(\frac{k_{12}}{k_{12} + k_{21}} \right) k_{23} \text{ if } k_{12}, k_{21} \gg k_{23} \qquad (3)$$

Because k_{23} is base-catalyzed under these conditions

$$k_{23} = k_b[OH^-] \qquad (4)$$

it follows that k_{obs} is base-catalyzed in this exchange mechanism, which is the EX2 mechanism of Hvidt and Nielsen [5].

Therefore, if an unfolding intermediate is in rapid equilibrium with N at the start of the unfolding process, it will show EX2 exchange, which is base-catalyzed. The problem then is to find out whether any rapidly formed unfolding intermediate contributes to the EX2 exchange shown in Fig. 1 for some RNase A NH protons. Initially, these EX2 exchange reactions, shown by various peptide NH protons, could not be understood. They appeared to be outside the bounds prescribed by the two-process model for EX2 exchange in proteins; this model was given independently in 1994 by Bai et al. [7] and by Qian et al. [8]. In the two-process model, exchange occurs either by small conformational fluctuations (process 1) or by global unfolding (process 2). Exchange by process 1 is observed to be denaturant insensitive, and it is overtaken at some critical denaturant concentration by exchange via process 2, whose rate increases rapidly with denaturant concentration.

An important prediction of the two-process model [7,8] is that EX2 exchange occurs only by global unfolding at denaturant concentrations beyond the midpoint of the unfolding transition zone, and all NH

Table 1
Protection factors of protons in RNaseA showing EX2 exchange before unfolding occurs [a]

Residue	P (pH 8) [b]	P (pH 9)
Glu 9	EX1 [c]	3.9×10^4
Met 30	2.6×10^4	1.3×10^5
Lys 31	EX1	4.2×10^4
Arg 33	7.8×10^3	$<10^4$
Asn 34	2.1×10^4	$<10^4$
Val 43	4.7×10^3	1.7×10^4
Asp 53	EX1	2.0×10^4
Ser 59	1.7×10^5	4.9×10^4
Gln 60	EX1	2.8×10^4
Lys 61	EX1	1.2×10^4
Cys 65	2.5×10^3	$<10^4$
Glu 86	EX1	7.1×10^4
Tyr 97	EX1	3.2×10^4
Cys 110	EX1	1.2×10^5
Val 124 [d]	20	<30

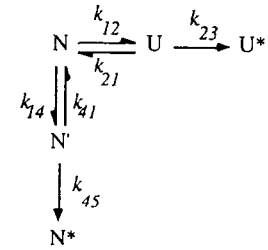
[a] Conditions: 10°C, pH 8.0 and pH 9.0, 4.5 M GdmCl [4].
[b] The protection factor P is calculated from $P = k_{23}/k_{EX2}$, where $k_{EX2} = k_{obs} - \langle k_{EX1} \rangle$. The values of k_{obs} are shown in Fig. 3 of [4] and the numerical values are given in Ref. [9]. The only cases considered are those where k_{obs} is faster than $\langle k_{EX1} \rangle$ by at least 3σ, where σ is the average standard error of the individual values of k_{obs}. Protons of Glu 2, Ala 6, Lys 7, Cys 26, Asn 27, Gln 28, Arg 33, Asn 34, and Asn 113 showed measurable exchange whose rates were too fast to measure accurately at pH 8, and they are not considered here.
[c] EX1, k_{obs} not statistically different than $\langle k_{EX1} \rangle$.
[d] Val 124, which is C-terminal, has an unusually small value of k_b [13], which explains its low protection factor but measurably slow k_{obs}.

protons show protection factors of value 1 at these denaturant concentrations. The protection factor P is defined by

$$P = k_b[OH^-]/k_{obs} \quad (5)$$

Both these predictions of the two-process model are strongly contradicted by the EX2 exchange results found for RNase A under unfolding conditions: the protection factors shown by various NH protons have diverse values unlike those seen in global unfolding, and they are large compared to 1 (see Table 1). Because exchange via unfolding intermediates is not included in the two-process model [7,8], it seemed possible at first that these EX2 exchange reactions are produced by rapidly formed unfolding intermediates. There is, however, a simpler and more straightforward explanation [9]. The fact that exchange via global unfolding follows the EX1 mechanism in the unfolding experiments of Kiefhaber and Baldwin [4] means that EX1 exchange must be included in a general two-process model. When this is done, the properties of the general model are quite different from those of the earlier two-process model [7,8] and the modified model has been termed the general two-process model [9] to avoid confusion.

The predicted exchange behavior of RNase A in the unfolding experiments of Kiefhaber and Baldwin [4] is shown in Fig. 2. It has been simulated by the model



(6)

In this model, exchange via small conformational fluctuations (process 1) occurs only by the EX2 mechanism, so that N′ is in rapid equilibrium with N and values for the rate constants k_{14}, k_{41} are not needed in the simulation. Exchange via global unfolding (process 2) may occur by either the EX1 or EX2 mechanism, depending on the relative values of k_{23} and k_{21}, k_{12}. The rate constants k_{45} and k_{23} both are assumed to be given by Eq. 4.

Fig. 2 shows that exchange via global unfolding is predicted to show a transition from the EX2 mechanism to the EX1 mechanism near 2 M GdmCl, below the unfolding transition zone. Fig. 2 also predicts that EX2 exchange occurs by small fluctuations at GdmCl concentrations beyond the unfolding transition zone and that the NH protons involved in these EX2 reactions have large protection factors. The general two-process model predicts correctly [9] that the rank order of the protection factors (Table 1) is the same as the rank order observed earlier for RNase A under non-denaturing conditions [10].

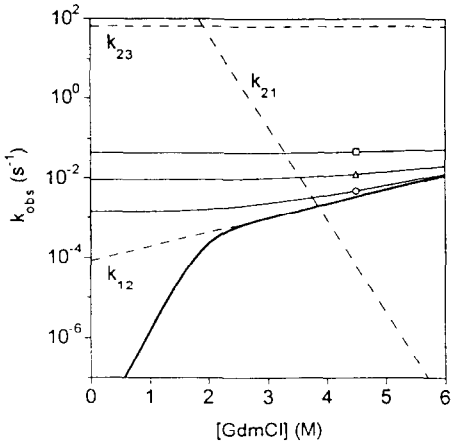


Fig. 2. Simulations of the exchange kinetics of RNase A at pH 8.0, 10°C, by the extended two-process model (figure reprinted from Loh et al. [9]). The bold line simulates exchange via global unfolding, which is predicted to show a transition from the EX2 exchange mechanism to the EX1 mechanism at about 2.2 M GdmCl. The thin lines simulate exchange via small conformational fluctuations for peptide NH protons Cys 65 (□), Arg 33 (△), and Met 30 (○). The unfolding rate constant k_{12} has been measured at 4.5 M GdmCl [4], and its dependence on the concentration of GdmCl is known. The refolding rate constant k_{21} was estimated [9] from results measured under other conditions, and the exchange rate constant k_{23} was estimated (see Eq. 4) from model peptide data [13]; k_{obs} is the predicted value of the observed rate constant for exchange. The exchange measurements of Kiefhaber and Baldwin [4] were made at 4.5 M GdmCl.

The reason is easily understood why EX2 exchange is still observed for some NH protons of RNase A under these unfolding conditions. Exchange occurs by the fastest pathway. The rate of EX2 exchange is $k_b[OH^-]/P$ (see Eq. 5). For sufficiently small values of the protection factor P, this EX2 rate is faster than k_{12}, the unfolding rate which gives the rate of EX1 exchange.

4. Limits on unfolding intermediates in rapid equilibrium with N

Suppose that a rapidly formed unfolding intermediate I is present under unfolding conditions; I may be either on-pathway or off-pathway.

$$N \rightleftarrows I \rightleftarrows U \qquad (7)$$

The observed protection factor, P_{obs}, for EX2 exchange from species other than U is

$$\frac{1}{P_{obs}} = \frac{f_N}{P_N} + \frac{f_I}{P_I} \qquad (8)$$

Suppose for a given peptide NH proton $P_I = 1$, so that exchange of this proton occurs chiefly from I and exchange from N can be neglected; then Eq. 8 becomes

$$\frac{1}{P_{obs}} \approx f_I \qquad (8a)$$

Because the smallest observed protection factor found for a peptide NH proton is 2500 (Cys 65, Table 1), the largest value of f_I for any possible unfolding intermediate is 4×10^{-4}. Either the possible unfolding intermediates are not significantly populated during the unfolding process, or else the 49 peptide NH protons that were studied are not affected by partial unfolding, or else the affected NH protons still have large protection factors. By definition, an unfolding intermediate should be partly unfolded during the unfolding process. The conclusion is, therefore, that possible unfolding intermediates with open H-bonds and protection factors near 1 are not observably populated during RNase A unfolding under these conditions. This is the same conclusion that was drawn earlier [4] for slowly formed intermediates that should be detected by EX1 exchange. It applies also now to rapidly formed intermediates that should be detected by EX2 exchange.

There are two caveats. The first caveat is that a partly unfolded intermediate may have sharply reduced protection factors in the affected part of the molecule, but these protection factors may nevertheless be large compared to 1. The properties of actual "partly unfolded intermediates" may be different from what one assumes. The second caveat is that the affected NH protons in a partly unfolded intermediate of RNase A might not be included among the 49 NH protons whose rates have been measured. For example, nine additional protons (Glu 2, Ala 6, Lys 7, Cys 26, Asn 27, Gln 28, Arg 33, Asn 34 and Asn 113) show detectable exchange at pH 8.0 that is too fast to be measured with the technique used [4]. In order to test for rapidly formed unfolding intermediates at the start of the kinetic unfolding process, one wants to compare the protection factors of all

possible NH protons with the ones measured in the native protein at 0 M GdmCl. The number of NH protons studied by Kiefhaber and Baldwin [4] is large (49), but probably the number can be increased substantially by using stopped-flow mixing to acquire data for fast-exchanging NH protons and by using the pulsed field gradient method to acquire 2D spectra rapidly.

Exchange rates of native cytochrome *c*, measured as a function of GdmCl concentration under sub-denaturing conditions, indicate that exchange occurs by more than the two processes specified in the earlier two-process model [11]. Bai et al. [11] have interpreted the results in terms of exchange from partly unfolded intermediates. In further work, it will be important to bring together these two methods of searching for unfolding intermediates to focus on a single protein.

5. Unfolding pathway of ribonuclease A

Any observable unfolding intermediates must precede the transition state for unfolding. RNase A appears to have a well-defined unfolding transition state. Monitoring the unfolding reaction by EX1 hydrogen exchange [4] shows that unfolding follows a single exponential curve whose rate is consistent with, although somewhat faster than, the unfolding kinetics monitored by CD or other optical probes. All measurable peptide NH protons undergo EX1 exchange during unfolding with the same rate. The probable reason for the difference between the unfolding rates measured by CD and by H-exchange has been discussed previously [4].

Despite the evidence that partly unfolded intermediates are not seen by H-exchange, a populated unfolding intermediate, monitored by the loss of chemical shift dispersion, has nevertheless been found by taking 1D ^{1}H NMR spectra in real time during unfolding [12]. The intermediate is formed rapidly at the start of the unfolding process. It appears to be a "dry molten globule": its side chains are somewhat disordered, but water apparently has not penetrated into the interior of the protein. Formation of this intermediate does not result in rapid hydrogen exchange of any of the 49 peptide NH protons studied [4]. The nature of this intermediate suggests that side chain interactions are not critical in forming the transition state for unfolding [4]. Instead, the entry of water into the interior of the protein is likely to be rate-determining for unfolding.

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