Effect of Mutation of Proline 93 on Redox Unfolding/Folding of Bovine Pancreatic Ribonuclease A[†]

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ABSTRACT: Both the reductive unfolding and oxidative regeneration of a P93A mutant and wild-type RNase A have been studied at 15 °C and pH 8.0. The rate of reduction of the 40−95 disulfide bond is accelerated about 120-fold by the P93A mutation, while the reduction of the 65−72 disulfide bond is not accelerated by this mutation (within the experimental error). Moreover, the reduction of native P93A to des[40−95] is about 10 times faster than the further reduction of the same des[40−95] species. These results demonstrate that the reduction of the mutant proceeds through a local unfolding event and provides strong support for our model in which the reduction of wild-type RNase A to the des species proceeds through two independent local conformational unfolding events. The oxidative regeneration rate of the P93A mutant is comparable to that of wild-type RNase A, suggesting that a cis 92−93 peptide group that is present in native wild-type RNase A and in native des[40−95], is not obligatory for the formation of the third (final) native disulfide bond of des[40−95] by reshuffling from an unstructured 3S precursor. Thus, the trans to cis isomerization of the Tyr92-Pro93 peptide group during the regeneration of wild-type RNase A may occur *after* the formation of the third native disulfide bond.

The kinetics of the unfolding/folding of bovine pancreatic ribonuclease A (RNase A),[1] a protein with four disulfide bonds and four prolines (two in the cis and two in the trans conformation), has been examined by two separate approaches in our laboratory. In one approach, the disulfides are reduced and the thiols are then oxidized with glutathione (*1*) or dithiothreitol (*2*−*4*). In the other approach, the disulfide-intact protein is unfolded with guanidine hydrochloride and then refolded by diluting this denaturation reagent (*5*−*8*).

The mechanisms for the DTT-induced unfolding and refolding are shown in Figures 1 (*9*) and 2 (*3*, *4*), respectively, where R, N, 1S, 2S, 3S, and 4S represent the reduced (R), native (N), the disulfide intermediate ensembles containing 1−4 disulfide bonds, respectively, and des[65−72] and des[40−95] represent RNase A lacking the 65−72 and the 40−95 disulfide bond, respectively. There are two major pathways, through the structured des[65−72] and des[40−95], respectively, in both the reduction (*9*) and regeneration

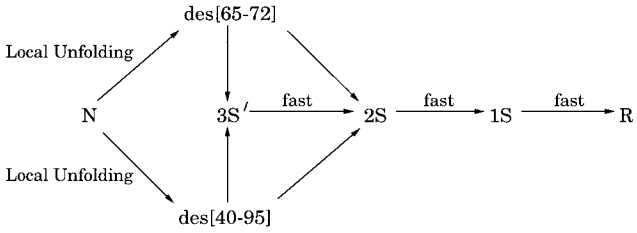

FIGURE 1: Kinetic model of the reductive unfolding of RNase A at 15 °C, pH 8 (*9*). R, N, 1S, 2S, and 3S′ represent the reduced (R), native (N), and rapidly interconverting disulfide ensembles containing 1−3 disulfide bonds, respectively.

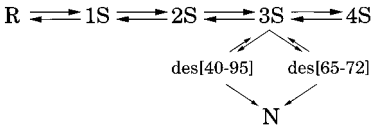

FIGURE 2: Oxidative regeneration model of RNase A at 25 °C, pH 8 (*4*), showing the major pathways. R, N, 1S, 2S, 3S, and 4S represent the reduced (R), native (N), and rapidly interconverting disulfide ensembles containing 1−4 disulfide bonds, respectively. The 3S reshuffling to des[40−95] and des[65−72] is the rate-determining step.

of wild-type RNase A (*3*, *4*). Other *minor* pathways in oxidative folding (*10*, *11*) are not considered in this discussion. The mechanism for the GdnHCl-induced unfolding and refolding of the disulfide-intact molecule is represented by the "box" model of Figure 3 (*8*), in which nonnative isomers of three prolines (Pro114, Pro117, and especially Pro93) retard the conformational folding.

An intriguing question is how are these processes coupled, i.e., which step occurs first, the conformational folding of the protein, the isomerization of the prolines to their native state, or the formation of the native disulfide bonds? We

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; DTT^{red}, DL-dithiothreitol; DTT^{ox}, oxidized dithiothreitol; GdnHCl, guanidine hydrochloride; AEMTS, 2-aminoethyl methanethiosulfonate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; 1S, 2S, 3S, and 4S, ensembles of rapidly interconverting disulfide species containing 1, 2, 3, and 4 disulfide bonds, respectively; des[26−84], des[58−110], des[40−95], and des-[65−72], RNase A lacking the 26−84, 58−110, 40−95, and 65−72 disulfide bonds, respectively; HPLC, high-performance liquid chromatography; P93A, a proline to alanine mutant at position 93 of RNase A.

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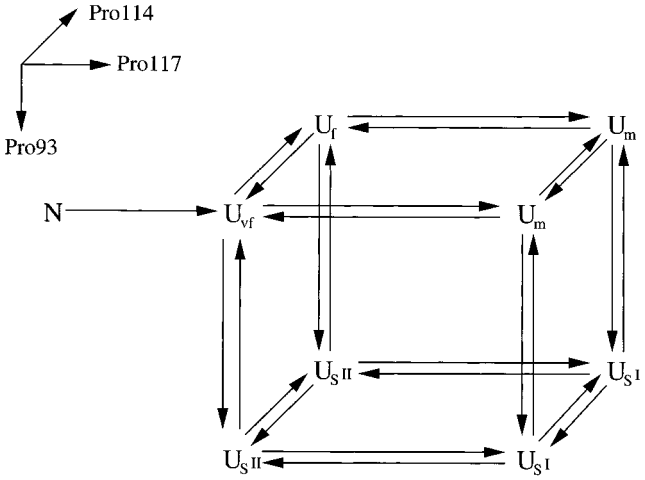

FIGURE 3: "Box" model for disulfide-intact reductive unfolding of RNase A (*8*). The protein unfolds conformationally from the native state (N) to the unfolded but very fast refolding species U_{vf}, in which only the native isomers of the X-Pro peptide groups are present. The fast, medium, slow, and very slow refolding species (denoted as U_f, U_m, U_{sII}, and U_{sI}, respectively) are then produced by independent isomerizations at the Asn113-Pro114, Val116-Pro117, and Tyr92-Pro93 peptide groups. The arrows above the figure indicate the prolines to which each dimension of the box corresponds, from which it is evident that nonnative isomers of Pro114, Pro117, and Pro93 cause increasing disruption of conformational folding. The Tyr92-Pro93 and Asn113-Pro114 peptide groups are cis in the native protein.

address this question here by investigating the kinetics of the DTT-mediated unfolding/folding of a mutant of RNase A, in which Pro 93 was replaced by alanine. The mutation decreases the stability of the protein, with its T_m lowered by about 12 °C (*7*), and perturbs the structure locally around residue 93 (*7*, *13*, *14*). Both the Tyr92-Pro93 and Tyr92-Ala93 peptide groups of native wild-type RNase A and the P93A mutant, respectively, are in the cis conformation (*7*, *12*−*14*). However, the isomerization rates and the equilibrium cis−trans ratio are quite different for an X-X compared to an X-Pro peptide group; this suggests that a study of the mutant and wild-type protein can provide information about the role of the trans to cis isomerization of Tyr92-Pro93 in oxidative folding. We focused on the question whether the isomerization of peptide group 93 to the native cis conformation in the precursor 3S species must precede the last disulfide reshuffling step that results in the formation of des-[40−95]. The stability and conformational folding/unfolding of this mutant have been studied extensively (*7*) and, together with the results of the reductive unfolding experiments in this study, which provide information about local/global fluctuations in the structured disulfide species, help in interpreting the oxidative folding data presented here.

MATERIALS AND METHODS

Wild-type RNase A was purchased from Sigma and purified as reported by Rothwarf and Scheraga (*2*). The P93A mutant of RNase A was expressed and purified as described by Dodge and Scheraga (*7*). AEMTS was prepared according to the method of Bruice and Kenyon (*15*). Ultrapure DTT^{red} was obtained from Sigma and used without further purification. DTT^{ox} was purchased from Sigma and purified by the method of Creighton (*16*). Reduced wild-type RNase A and reduced P93A mutant were prepared and purified as described previously (*2*). All other chemicals were of the highest grade commercially available.

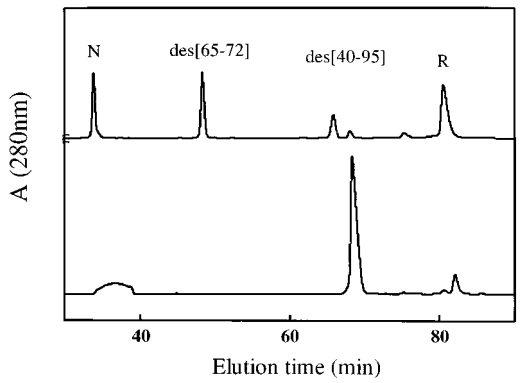

FIGURE 4: Chromatograms from a 36 h reduction mixture of wild-type RNase A (top) and a 1 h reduction mixture of the P93A mutant (bottom). Conditions for both reductions were pH 8.0, 15 °C, 100 mM DTT^{red}, and 80 μM protein. The reduced protein is designated R, and the native protein is N. The peak for N in the bottom chromatogram is very broad.

The reduction procedure was similar to that used for wild-type RNase A (*9*). The initial reduction conditions were 80 μM P93A in 100 mM Tris-HCl and 1 mM EDTA buffer at pH 8 and 100 mM DTT^{red}, degassed by humidified argon gas, and the temperature was controlled at 15 °C. At various times, a 0.5 mL reaction aliquot was removed and blocked by adding 100 mg of solid AEMTS for 2 min and then quenched by adding 25 μL of acetic acid. After being desalted by elution on a G25 superfine column with 50 mM acetic acid solution, the sample was loaded onto a Hydropore SCX (Rainin) analytical HPLC column.

The oxidative regeneration process was carried out as described by Rothwarf and Scheraga (*2*), with minor modification. The initial oxidation conditions were 20−32 μM protein in 100 mM Tris-HCl and 1 mM EDTA buffer at pH 8 and 50−100 mM DTT^{ox}, degassed by humidified argon gas, and the temperature was controlled at 15 °C. At various times, a reaction aliquot was removed and blocked by AEMTS solution (final concentration of 3 mM) for 2 min and then quenched by adding acetic acid. After being desalted by elution on a G25 superfine column with 50 mM acetic acid solution, the sample was loaded onto a Hydropore SCX (Rainin) analytical HPLC column. A 30 s DTT^{red} (final concentration 10 mM) reduction pulse was also carried out to identify the peaks of the stable des species (*4*). This DTT^{red} concentration is sufficient to reduce all of the unstructured intermediates to the reduced protein.

RESULTS

Figure 4 compares the chromatograms from a 36 h reduction of wild-type RNase A with that from a 1 h reduction of the P93A mutant, under the same reducing condition (at 15 °C and pH 8.0). The major difference between these two reductions is that, whereas des[65−72] is the dominant intermediate for wild-type RNase A, des-[40−95] is the dominant intermediate in the reduction of P93A with almost no des[65−72] being observed. The lag time between the appearance of intermediates (des species) and that of the fully reduced species is also significantly shortened, from about 3 h for wild-type RNase A (*9*) to about 10 min for the P93A mutant.

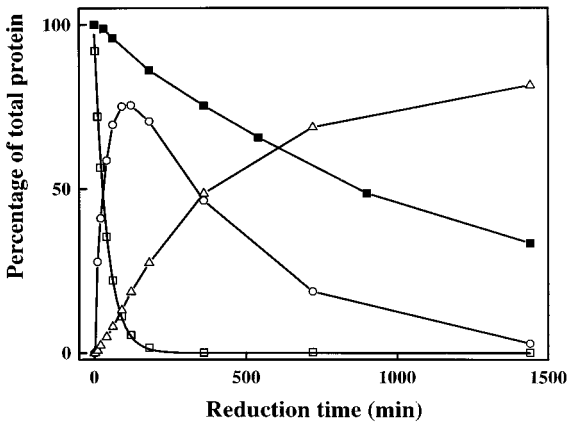

FIGURE 5: Relative concentrations of species as a function of reduction time (at 15 °C, pH 8.0, 100 mM DTT^{red}, 80 μM protein). Plots: (□) native P93A; (○) des[40−95] of P93A; (△) reduced P93A; (■) native wild-type RNase A. The decay of native P93A is describable by a single-exponential curve.

Table 1: Reduction Rate Constants of RNase A at 15 °C and pH 8

	wild-type RNase A[a]	P93A mutant	P93A/ wild-type
native → des[40−95] (min^{-1} M^{-1})	0.0021	0.26	124
des[40−95] → 2S (min^{-1} M^{-1})	0.0068	0.024	3.5

[a] Data from Li et al. (*9*).

The reduction of P93A is significantly faster than that of wild-type RNase A. Within 3 h, more than 98% of native P93A has been reduced, mostly to des[40−95], and the amount of des[65−72] is unobservable within experimental error (Figure 5). Assuming that the reduction rate is first order in DTT^{red} (*9*), the decay curve of native P93A versus time could be fitted to a single-exponential function with a rate constant of 0.26 min^{-1} M^{-1} for reduction of its 40−95 disulfide bond. This is about 120-fold larger than that of wild-type RNase A (0.0021 min^{-1} M^{-1}) (*9*). Moreover, the decay curve of des[40−95] of the mutant (after about 250 min when the amount of native P93A has become negligible) can be fitted to a single-exponential function with a rate constant of 0.024 min^{-1} M^{-1} for the reduction of des[40−95] of P93A (Table 1). It is worth noting that the reduction rate of native P93A to des[40−95] is about 10-fold faster than the rate of further reduction of des[40−95].

Figure 6 shows that, during oxidative regeneration at 15 °C and pH 8.0, both the P93A mutant and wild-type RNase A exhibit similar 1S and 2S groupings but a difference in the 3S region. Figure 7 compares the chromatograms for wild-type and P93A RNase A after a 30 s reduction pulse at 15 °C and pH 8.0. As with wild-type RNase A, the des[40−95] species is the dominant 3S species during the regeneration of the P93A mutant of RNase A; however, the three other des species, which are observed in the regeneration of the wild-type protein (*17*), are not observable in the regeneration of P93A. From Figure 8, it can be seen that the regeneration rate of the native protein and the disappearance rate of the fully reduced P93A mutant are comparable to those of wild-type RNase A.

The native peak of P93A in Figures 4 and 6 is much broader than that of wild-type RNase A. This is because P93A is less stable than wild-type RNase A (*7*) and is denatured during the desalting process due to the low pH of the eluting solution (50 mM acetic acid); as a consequence, native P93A may be somewhat unfolded during the elution on the HPLC column. This is demonstrated by using a neutral solution to desalt, which produces a much sharper native peak, although still a little broader than that of wild-type RNase A. In addition, at a late stage of reduction (more than 20 h), the accumulation of perhaps some byproducts in the 1S region becomes significant [small peak near R after 1 h, in Figure 4, and a larger peak after 24 h (not shown here)]; hence the sum of the native, des[40−95], and reduced forms of P93A is less than 100% in Figure 5 by the amounts of byproducts in the 1S region.

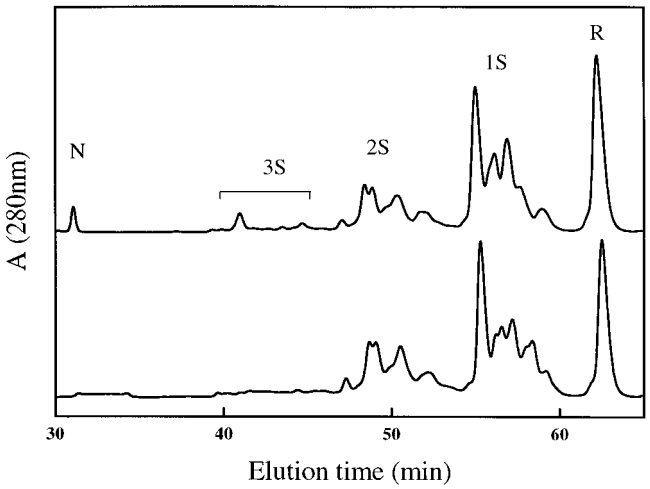

FIGURE 6: Chromatograms from a 3 h oxidative regeneration mixture of wild-type RNase A (top) and the P93A mutant (bottom). Conditions for both regenerations were pH 8.0, 15 °C, 100 mM DTT^{ox}, and 32 μM protein. The peak for N in the bottom chromatogram is very broad.

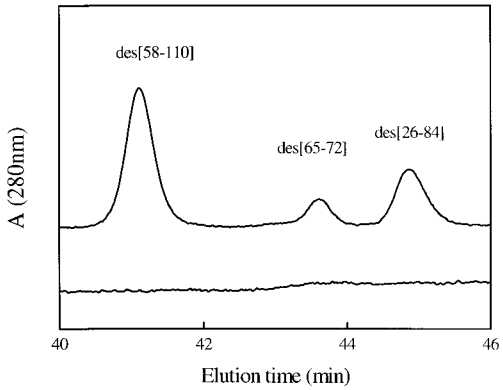

FIGURE 7: Chromatograms of the three-disulfide region of the same oxidative regeneration mixture as in Figure 6, which had been subjected to a 30 s pulse of DTT^{red} prior to blocking with AEMTS. Plots: (top) wild-type RNase A; (bottom) P93A. The large peak for des[40−95] of both wild-type RNase A and the mutant is not shown in the figure because it appears in the region of the 1S peak (*3*).

DISCUSSION

Reduction of RNase A. Kinetic studies (*9*) have suggested that the mechanism for the reduction of wild-type RNase A at 15 and 25 °C, pH 8, is analogous to the EX2 mechanism of amide proton exchange (*18*, *19*) in which the first step involves structural unfolding followed by reduction of the exposed disulfide bond. The reaction may be represented (*9*) as

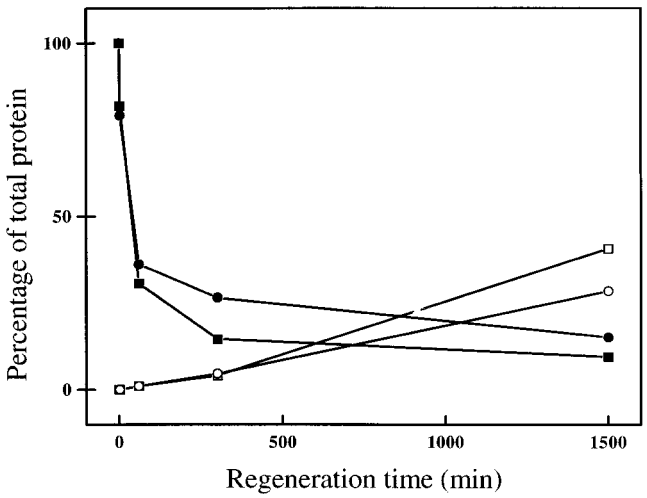

FIGURE 8: Relative concentration of species as a function of oxidative regeneration time (pH 8.0, 15 °C, 50 mM DTT^{ox}, 20 μM protein). Plots: (□) native P93A mutant; (○) native wild-type RNase A; (■) reduced P93A mutant; (●) reduced wild-type RNase A.

$$N \underset{k_u}{\overset{k_f}{\rightleftarrows}} N_u \rightarrow I \qquad (1)$$

where N is the native protein, N_u is a *locally* unfolded species (where k_f and k_u are the rate constants for local conformational folding and unfolding, respectively), and I is a des species, i.e., des[40−95] or des[65−72]. Reduction of one more native bond unfolds the protein and leads to the formation of the reduced species by fast, successive reduction steps without accumulation of any other intermediates (Figure 1). The only two intermediates populated on reductive unfolding of the wild-type protein at 15 °C are two structured des species, des[65−72] and des[40−95]. The other two des species, des[26−84] and des[58−110], are not populated on *reductive unfolding* of wild-type RNase A, primarily because the reduction rates of the 26−84 or 58−110 bonds whose exposure requires global unfolding are much less than those for the 65−72 and 40−95 bonds which can be exposed by local unfolding. Thus, the rate-determining steps involve two independent local unfolding events near the 65−72 and 40−95 disulfide bonds, respectively, without a common transition state (*9*). The data presented here further support this view, as indicated below.

The reduction rate of the 40−95 bond of native P93A is about 10 times faster than the reduction rate of des[40−95] of P93A (Table 1), clearly indicating that the first step in the reduction of P93A is a local and not a global unfolding event. The reduction rates of the 65−72 and 40−95 bonds of the native wild-type protein are similar (*9*). However, in native P93A, the reduction rate of 40−95 is at least 10 times faster than that of the 65−72 bond, judging from the negligible population of des[65−72] before the appearance of any further reduced species while a significant amount of native P93A has been reduced to des[40−95] (Figure 5). The mutation selectively accelerates the reduction of the 40−95 bond, supporting the earlier contention (*9*) that there are separate transition states for the two local unfolding events.

The reduction rate of native P93A is 120 times greater than that of the native wild-type protein. However, the reduction of des[40−95] of P93A is only 3 times that of des[40−95] of wild-type RNase A (Table 1). Thus, the data indicate that the perturbation caused by the absence of the 40−95 disulfide bond and by the P93A mutation is not additive. NMR studies (*12*) have shown that [C40A, C95A] and wild-type RNase A have similar structure, with the main difference being in the region around the missing 40−95 disulfide bond. X-ray diffraction (*13*) and further NMR (*14*) studies show that, although P93A retains the cis conformation of wild-type RNase A, the two proteins differ in structure, primarily around position 93. Thus, the structural perturbation (and destabilization) by the P93A mutation and by the breaking of the nearby 40−95 disulfide bond occurs locally, in similar regions of the molecule; this is consistent with the nonadditivity described above.

Oxidative Regeneration of RNase A. Cation-exchange chromatography of the AEMTS-blocked sample enables the intermediates to be separated according to the number of their disulfide bonds. Most of the intermediates of wild-type RNase A [R, 1S, 2S, 3S (except for the des species), and 4S] do not have stable tertiary structure, as indicated by their fast reduction rate [some 100 000-fold larger than the reduction of the des species and native RNase A (*4*)], but they may have some local structure around the 65−72 bond as demonstrated by the nonrandom distribution of one-disulfide bonds in the 1S (*20*) and 2S (*21*) ensembles. Des-[65−72] and des[40−95] of wild-type RNase A have stable nativelike structure as shown by NMR experiments on des species (*22*) and their mutant analogues (*12*, *23*, *24*); they also have similar thermal stabilities, with the T_m of the des-[65−72] analogue (44 °C) being slightly higher than that of the des[40−95] analogue (42 °C) (*25*). Recently, two new des species, des[58−110] and des[26−84], also shown in Figure 7, were found during the *oxidative regeneration* of wild-type RNase A at 15 °C and pH 8.0 (*17*). These two des species may not be stable at 25 °C but are sufficiently structured at 15 °C to withstand a reduction pulse (*17*).

The most obvious difference between the oxidative folding of P93A and the wild-type protein is in the 3S region of the chromatogram (Figure 6), indicating an altered distribution of the intermediates. Three of the des species, des[26−84], des[58−110], and des [65−72], elute in the 3S region of the chromatogram (*17*). (Des[40−95] elutes in the 1S region of the chromatogram.) The reduction-pulse experiment, in which all of the unstructured intermediates are reduced to R but the structured ones are not, suggests that the difference between the 3S profile of the wild-type and mutant chromatogram is in the population of the structured species. Des-[26−84], des[58−110], and des[65−72] of P93A do not withstand a reduction pulse and do not appear to be populated during the regeneration of P93A (Figures 6 and 7). The absence of a disulfide bond in these des species destabilizes different regions of the protein than the one(s) destabilized by the P93A mutation. Thus, the mutation likely makes these des species unstable at 15 °C and provides an explanation for the absence of des[26−84] and des[58−110] in the regeneration of P93A. The more stable des[65−72] of P93A is expected to be stable at 15 °C. However, the reductive unfolding experiment has shown that the local destabilization around the 40−95 disulfide bond leads to a considerably increased reduction rate which is likely responsible for the absence of des[65−72] in the chromatogram after a reduction pulse.

The rate of formation of the native form of P93A is comparable to that of the wild-type protein. At 15 °C, about 30% of the wild-type protein regenerates through des[26−84] and [58−110] (*26*). These intermediates are long-lived kinetic trapped species which, eventually, reshuffle to other intermediates rather than oxidize directly to the native form (*26*). An increased regeneration rate of P93A might have been expected due to the destabilization by the P93A mutation of the kinetically trapped species. However, after initiation of the reoxidation of the reduced protein, a quasi-equilibrium is established among the (R, 1S−4S) intermediates (*27*, *28*). The structured intermediates (the des species) are not part of the quasi-equilibrium and emerge slowly from the 3S ensemble by intramolecular reshuffling (*4*, *26*−*28*). Therefore, the destabilization of the kinetic trapped species, which may prevent their accumulation during the regeneration of P93A, has no direct effect on the regeneration rate except that it increases the concentration of all the unstructured ensembles which participate in the quasi-equilibrium. Thus, in the regeneration of the mutant (as compared to the wild type) protein, a small increase in the concentration of R makes the disappearance of R seem slightly slower, and a small increase in the concentration of 3S increases the rate of formation of the native form. However, these effects are similar in magnitude to the error of the experiment. The pathway involving des[40−95] eventually accounts for more than 90% of the wild-type protein regenerated at 15 °C (*26*). Thus, the comparable rate of regeneration of the wild-type protein and P93A suggests that the mutation does not have much influence on the formation of des[40−95] from 3S.

The central question in our study is how conformational folding, proline isomerization, and disulfide reactions are coupled in oxidative regeneration. While it is not possible to discern the mechanism of the coupling from these experiments, these data rule out one possible mechanism, as shown below, and suggest that isomerization of the 93 peptide bond to native can occur after formation of all of the three disulfide bonds of des[40−95]. Since the intermediates of RNase A, except for the des species, are unfolded at 15 °C, the coupling among conformational folding, proline isomerization, and disulfide reactions can occur in the last intramolecular reshuffling reactions in the precursor 3S species, leading to formation of the third (final) native disulfide bond of des[40−95] with concomitant folding of this species. Theoretically, 12 different precursors can exist in the regeneration mixture, each having two native disulfide bonds and one nonnative one. In the unfolded precursor of the wild-type protein, proline 93, which has a large effect on the conformational folding rate (*7*), is in either the cis or trans conformation. However, in the unfolded P93A mutant protein, peptide group 93 is only in the nonnative trans conformation. Thus, the close agreement between the regeneration rate of the wild-type and the mutant protein allows us to distinguish between two possible models. In the first model, the formation of the third native disulfide bond of des[40−95] in one or more precursor species can occur only if the species has a native cis peptide group 93. In the precursor species with a trans peptide group 93, this reaction does not occur at all or occurs only at a much lower rate. This model suggests that conformational folding in the precursor species with the obligatory native cis peptide group precedes the formation of the third native disulfide bond. In the second model, formation of the third native disulfide bond in the precursor species can occur with either native cis or nonnative trans peptide group 93 at a similar rate. This model is consistent with conformational folding occurring either before or after the formation of the third native disulfide bond in des[40−95]. The unfolded protein with the P93A mutation has practically no native cis peptide group 93, and thus, its regeneration rate would be drastically decreased if the first model were valid. However, the mutation would have only a small effect on the regeneration rate if the second model were valid. The close agreement found between the regeneration rate of the wild-type and P93A protein suggests that the second model is more likely.

CONCLUSIONS

Our results show that the effect of the P93A mutation on the reduction of RNase A is mainly local; the reduction rate of the 40−95 disulfide bond is accelerated about 120 times, while considerably less acceleration is observed for the reduction of the 65−72 disulfide bond. There is no significant difference between the oxidative regeneration rates of the P93A mutant and wild-type RNase A, showing that cis/trans isomerization about the Tyr92-Pro93 peptide group is not the rate-determining step in the regeneration of wild-type RNase A under the conditions used here and suggesting that the Tyr92-Pro93 trans to cis isomerization during the oxidative regeneration of wild-type RNase A can take place after the formation of three native disulfide bonds.

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