Regeneration of Bovine Pancreatic Ribonuclease A: Identification of Two Nativelike Three-Disulfide Intermediates Involved in Separate Pathways[†]

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Received November 18, 1997

ABSTRACT: During the regeneration of bovine pancreatic ribonuclease A (RNase A) from the reduced to the native form with mixtures of oxidized and reduced dithiothreitol at 25 °C, pH 8.0, the disulfide-containing protein intermediates achieve a steady-state distribution. By manipulating the redox conditions after the attainment of the steady-state condition, it has been possible to kinetically trap and, thereby, isolate and identify the disulfide-bonded species that follow the rate-determining step in the regeneration pathway. Two three-disulfide species have been identified by peptide mapping. Both species contain three native disulfide-bond pairings, one lacks the 65−72 disulfide bond (des-[65−72]), and the other lacks the 40−95 disulfide bond (des-[40−95]). These species are the same as those identified during the reduction of RNase A. By restarting the regeneration process from isolated des-[65−72] and des-[40−95], it is shown that both intermediates lie directly on regeneration pathways.

The sequence of structural steps by which a protein regenerates from its reduced and unfolded state to its fully oxidized native form is an intriguing puzzle, which has confounded solution since Anfinsen (*1*) first showed that the amino acid sequence contains all of the necessary information to direct this refolding process. In the regeneration procedure, the formation of disulfide bonds is controlled by the type and concentration of the thiol/disulfide redox reagent used to carry out the oxidation process. Both linear (e.g., cystine, glutathione, etc.) and cyclic disulfide reagents have been used to reoxidize proteins (*2*). Cyclic disulfides such as oxidized dithiothreitol (DTT^{ox})[1] have the advantage that they do not form stable mixed disulfides with protein thiols because of their rapid rate of recyclization when involved in mixed disulfides. This greatly simplifies the investigation of the regeneration process. For this reason, the studies presented here were carried out with the cyclic disulfide DTT^{ox}. However, even with the use of DTT^{ox} and the advantages of being able to exert thermodynamic control over the oxidation/reduction process by manipulation of the thiol/disulfide redox ratio, the problem is still very difficult, and only limited attempts have been made to characterize the regeneration pathways of proteins. The complexity of the regeneration problem increases rapidly with the number of disulfide bonds in the protein. Most regeneration studies have been carried out on proteins containing three or less disulfide bonds (*3*−*10*), although recently some progress has been made in the description of the regeneration pathways of the four-disulfide protein α-lactalbumin (*11*, *12*).

In a series of papers (*13*−*16*), the first steps of a general method to elucidate regeneration pathways were applied to the four-disulfide protein bovine pancreatic ribonuclease A (RNase A). This general method is well suited to the more complex problems encountered during the regeneration of proteins containing four or more disulfide bonds. In this method, groups of intermediates are divided according to the number of intramolecular protein disulfide bonds that they contain rather than to their specific disulfide-bond pairings. In the case of a four-disulfide protein, this translates into considering five groupings of intermediates, i.e., reduced protein (R), one-disulfide (1S), two-disulfide (2S), three-disulfide (3S), and four-disulfide (4S) species, rather than all 763 possible disulfide-bonded intermediates. The theoretical and experimental justifications for this type of analysis have been discussed extensively (*13*−*15*, *17*) and will not be repeated here. It is possible to group the intermediates in this way because the rate of interconversion among species within a group is more rapid than the interconversion between groups. Such a situation is expected when DTT^{ox}/DTT^{red} is used as the redox couple because the rates of oxidation and reduction, which involve intermolecular processes, are much slower than the rates of intramolecular disulfide rearrangement that occurs within groupings (*14*, *15*).

Applying this method to RNase A, the types of regeneration pathways and the kinetics of the oxidation, reduction, and disulfide rearrangement of groupings of intermediates were determined quantitatively (*13*, *14*). From such information, it was possible to account quantitatively for the concentrations of groupings of intermediates and the forma-

[†] This work was supported by Grant GM-24893 from the National Institute of General Medical Sciences of the National Institutes of Health. Support was also received from the National Foundation for Cancer Research.

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor; des-[40−95], RNase A lacking the 40−95 disulfide bond; des-[65−72], RNase A lacking the 65−72 disulfide bond; AEMTS, 2-aminoethyl methanethiosulfonate [$(NH_2)-C_2H_5-S-SO_2-CH_3$]; DTT^{ox}, oxidized dithiothreitol; DTT^{red}, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GdnHCl, guanidine hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HPLC, high-performance liquid chromatography; TEAA, triethylammoniumacetate; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

S0006-2960(97)02822-5 CCC: $15.00 © 1998 American Chemical Society
Published on Web 02/25/1998

tion of native protein as a function of time and redox reagent concentration. It was observed that the various groupings of intermediates achieved a preequilibrium or steady-state condition. This information was then used in a detailed kinetic analysis to determine the nature of the rate-determining step. This analysis considered all possible types of rate-determining steps. It was shown that the rate-determining step in the formation of native RNase A involved rearrangement of one or more three-disulfide species. This was expressed by the simple equation, 3S → 3S*, where 3S denotes the *entire* grouping of three-disulfide species except for 3S*, and 3S* represents those species formed *after* the rate-determining step. The identification of the specific type of rate-determining step(s) is the first step in a general approach to study protein regeneration. In this paper, the second step of this general procedure, the identification of the specific intermediates that follow the rate-determining step, will be applied to the study of the regeneration pathways of RNase A to identify the 3S* species.

The process by which 3S oxidizes to the native protein, N, may be expressed by the following two reactions:

$$3S \rightarrow 3S^* \quad (1)$$

$$3S^* + DTT^{ox} \rightarrow N \quad (2)$$

The first reaction (eq 1), which is the rate-determining one, is a rearrangement step and has no redox dependence. The second reaction (eq 2) is dependent on the concentration of DTT^{ox}. Therefore, if at some point after the formation of the 3S grouping the redox reagent is removed, i.e., no DTT^{ox} is present, then the reaction shown in eq 1 will proceed and 3S* will form, but the reaction in eq 2 is blocked and 3S* cannot oxidize to native. Therefore, 3S* should accumulate. Here, we present the results of carrying out this procedure.

Two separate 3S* species have been identified. Both contain three native disulfide bonds. One species lacks the 40−95 disulfide bond and will be referred to as des-[40−95]. The other species lacks the 65−72 disulfide and will be referred to as des-[65−72]. Some of the properties of these intermediates as well as a qualitative analysis of the role they play in the regeneration process are presented. A more detailed and quantitative analysis of the two pathways as a function of redox conditions is provided in the accompanying paper (*18*).

MATERIALS AND METHODS

Materials. Native and reduced RNase A (type 1-A, Sigma) were prepared as described previously (*13*). DTT^{red} ultrapure was obtained from Boehringer-Mannheim. DTT^{ox} (Sigma) was purified by the method of Creighton (*19*). AEMTS was synthesized as described by Bruice and Kenyon (*20*). Trypsin (type III) and α-chymotrypsin (type II) were purchased from Sigma and used without further purification. All other reagents were of the highest grade commercially available.

Regeneration of RNase A. The regeneration process was initiated by the addition of a concentrated solution of the reduced protein to a solution of 100 mM Tris and 2 mM EDTA, pH 8.0, containing 200 mM DTT^{ox} under argon. The initial concentration of reduced protein was 10−32 μM. All concentrations were determined using the extinction coefficient previously reported (*13*). The regeneration mixture was maintained under a continuous flow of humidified argon. All experiments were carried out at 25 °C. The temperature was regulated to ±0.1 °C by use of a jacketed bath connected to a Haake F-1 circulating bath. After 50 min, an aliquot was removed, and the unreacted thiols were blocked by the addition of 10 mM AEMTS freshly dissolved in 100 mM Tris, and 2 mM EDTA, pH 8.0. After 2 min, the blocked protein mixture was diluted with 125 mM TEAA pH 5 buffer. The diluted protein solution was then either immediately injected onto an HPLC column as described below or frozen at −70 °C until such time as it could be analyzed by HPLC. Within experimental error, there was no difference between the chromatograms produced from the samples treated in these two procedures.

Fractionation of Intermediates. Intermediates were fractionated by HPLC cation-exchange on a Rainin Hydropore SCX 4.6 mm × 10 cm column. Samples were not desalted prior to injection onto the ion-exchange column. A ternary gradient was used both to desalt and to fractionate the intermediates. Samples were injected onto the column, which was equilibrated with 50 mM acetic acid, pH 5.0. A gradient to 50 mM NaCl was then used to elute unreacted AEMTS, DTT^{ox}, and AEMTS-blocked DTT^{red}. No intermediates washed off during this procedure, and the optimum conditions were easily determined from control experiments using native RNase A, since the native protein is the least charged and, therefore, the most weakly retained of any of the species under all chromatographic conditions. Once the salts had been eluted, the column was reequilibrated at pH 7.0 in 25 mM HEPES buffer and 1 mM EDTA, and the NaCl gradient reported previously (*21*) was run. A Spectra-Physics SP8700 gradient system or an LKB 2249 gradient pump was used. The detector, an ISCO UA-5 type 9 optical unit and 280 nm filter, was interfaced to an a/d convertor, and the data were stored on a Sun IPC computer.

The temperature of the column was regulated in some experiments by placing the column in a circulating bath. A precolumn stainless steel coil was also placed in the bath to enhance thermal equilibration of the buffers. A postcolumn heat exchanger was employed to minimize detector noise.

Rearrangement Studies. The 5 mL aliquots were removed from the regeneration mixture at 50 min, and the regeneration process was quenched by the addition of sufficient 1 M HCl to drop the pH to 2. These samples were immediately desalted on a Pharmacia HR16/50 column packed with Sephadex G-25 superfine resin (Pharmacia). The samples were desalted into 100 mM acetic acid at 25 °C and lyophilized. Recovery was better than 90%. The delivery and detection systems consisted of an LKB 2150 pump with a Rheodyne 7125 injector and an ISCO UA-5 detector with type 9 optical unit and 280 nm filters.

The rearrangement process was carried out in 100 mM Tris, and 2 mM EDTA, pH 8.0, buffer at 25 °C starting from the lyophilized HCl quenched material. At various times (1−1200 min), aliquots were removed, blocked with AEMTS, and analyzed by HPLC as described above.

To ensure that the pH quenching and lyophilization processes were not affecting the results of the rearrangement studies control experiments were carried out. In these experiments, the pH was not quenched but the sample was immediately injected onto the same desalting column con-

taining 100 mM Tris and 2 mM EDTA, pH 8.0, which was being continuously sparged with argon. Within experimental error, there was no difference in the results obtained using these two procedures.

Rearrangement studies were also carried out in 100 mM Tris, 2 mM EDTA, pH 8.0, buffer at 25 °C, starting from purified des-[65−72] and des-[40−95] isolated from reduction of native RNase A as described in the following subsection.

Direct Oxidation Measurements. Two methods were used to measure the rate of oxidation of des-[65−72] and des-[40−95] to native protein. In the first method, des-[65−72] and des-[40−95] were obtained in their unblocked form by selective reduction of the native protein and purified by a multistep procedure. The selective reduction of the protein was carried out as follows: the native protein was dissolved in 100 mM Tris, pH 8.7 (at 25 °C), and equilibrated at 10 °C prior to the addition of a stock solution of DTT^{red} to yield final conditions of 10−20 mg/mL of RNase A and 400−500 mM DTT^{red}. After 6−10 h, the protein was desalted on a Pharmacia HR16/50 column packed with Sephadex G-25 superfine resin (Pharmacia) into 25 mM acetic acid at 25 °C and lyophilized. The lyophilized protein was dissolved in 50 mM acetic acid, pH 5.0, and the intermediates were fractionated by HPLC cation-exchange on a Rainin Hydropore SCX 21.0 mm × 10 cm column using a NaCl gradient from 60 to 160 mM over 40 min. The buffer system used was 50 mM acetic acid and 1 mM EDTA, pH 5.0, at a flow rate of 8 mL/min. A Spectra-Physics SP8700 gradient system was used. A final purification was carried out at pH 2 on a reverse phase column using procedures described previously (*22*). The purified material was lyophilized and stored desiccated in vacuo. Purity was checked by adding lyophilized material directly to AEMTS and analyzed by cation exchange HPLC as described above. Using these procedures des-[65−72] and des-[40−95] were obtained in their unblocked form at >98% purity. To minimize disulfide rearrangement during the purification procedures, all fractions collected off of the various columns were kept on ice.

The lyophilized species were denatured under the conditions used in the final stage of their purification. Therefore, when they were dissolved directly in a pH 8 buffer, significant disulfide rearrangement occurred. This is expected since the rate of disulfide bond rearrangement in unfolded RNase A derivatives (*14*) is expected to be comparable to the rate of conformational folding (*23*). Consequently, the lyophilized protein was initially dissolved in ice cold 50 mM acetic acid, pH 5, and equilibrated on ice. The lower pH slows down the rate of disulfide-bond rearrangement but does not significantly affect the rate of conformational folding. After 25−30 min, the solution was allowed to equilibrate at 25 °C. The pH was increased to 8 by the addition of an appropriate amount of 200 mM Tris buffer, and DTT^{ox} was added to initiate the oxidation process. The final protein concentration was 20 μM. Aliquots were removed at various times, blocked, and analyzed using cation-exchange HPLC as described above. Relatively high concentrations of DTT^{ox} (50−150 mM) were used in these experiments to minimize effects due to rearrangement and air oxidation.

A second and more direct method was also used to measure the rate constant for the oxidation of des-[65−72] and des-[40−95] to form the native protein. In this method, DTT^{ox} was added to the regeneration mixture, which had been allowed to rearrange in the absence of redox reagent for 400 min. Aliquots were removed at various times, blocked, and analyzed using cation-exchange HPLC as described above.

All experiments were carried out at 25 °C. To minimize the contribution from air oxidation, all equilibrations were carried out under a humidified argon atmosphere, and all buffers contained 2 mM EDTA.

Evaluation of Desalting Procedures. To test whether the use of the acrylamide based desalting columns resulted in the preferential loss of one of the nativelike three-disulfide species, a regeneration mixture containing known amounts of des-[65−72] and des-[40−95] was passed over disposable 10DG columns (Bio-Rad) into 50 mM TEAA, pH 5.0, at 25 °C using the procedures from our earlier study (*13*). Both the protein and salt-containing peaks were saved for HPLC analysis as described above.

Peptide Mapping. Because of the resistance of native and the nativelike RNase A species (especially des-[65−72]) to enzymatic digestion, two different approaches were taken. In one case, the proteins (7 mg/mL) were digested for 100 min by chymotrypsin (1/50 mass ratio) in 100 mM Tris, 2 mM EDTA, and 2 M GdnHCl at pH 7.6 and 37 °C. The GdnHCl was removed by desalting into 0.09% TFA on a Waters Novapak C18 column using acetonitrile as the eluent. The desalted partially digested protein was then lyophilized and resuspended to a concentration of 7 mg/mL in 100 mM Tris and 2 mM EDTA, pH 7.6 and 37 °C, and digested for 100 min with Trypsin (1/50 mass ratio). The digestions were stopped by dropping the pH to 2 with TFA. This procedure was applied to native as well as des-[65−72]. The inclusion of GdnHCl in this procedure permitted complete digestion of native RNase A. The digestion of native RNase A served as a control to exclude possible complications that could arise as a result of the native disulfide-bond pairings, e.g., incomplete digestion or disulfide rearrangement. However, no such complications were encountered. A second procedure, with GdnHCl omitted, was applied to des-[65−72] and des-[40−95] and is described elsewhere (*22*).

In addition to UV absorbance, the disulfide detection system of Thannhauser et al. (*24*) was used to analyze the peptide maps with the modifications described previously (*13*). A Spectra-Physics SP8800 gradient system with Gilson UV116 variable wavelength detector with a Waters Novapak C_{18} column and an ABI model 130A microbore system with a YMC C_{18} minibore column were used for peptide mapping. All the data were digitized and stored on a Sun IPC computer. Disulfide-containing peaks were collected and were identified by amino acid analysis and/or determination of their molecular mass by measurement on a matrix assisted laser desorption mass spectrometer.

Peptide-mapping identification was carried out on des-[65−72] and des-[40−95] isolated from reduction (*21*, *22*) and from the regeneration process. There was no significant difference between the peptide maps carried out on intermediates isolated from reduction mixtures and those isolated from regeneration mixtures. In the case of the regeneration, des-[65−72] and des-[40−95] were separated from the other regeneration intermediates by two rounds of cation exchange

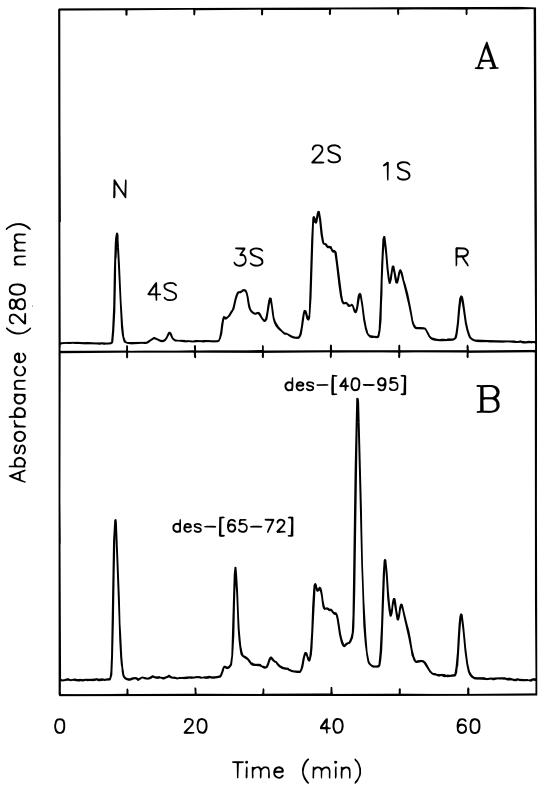

FIGURE 1: Chromatograms showing the rearrangement experiment. (A) A typical regeneration mixture, 200 mM DTT^{ox}, 32 μM RNase A, pH 8.0, 25 °C, 50 min. (B) The same mixture that had been desalted to remove DTT^{ox} and DTT^{red}, as described in the text, and allowed to equilibrate for an additional 120 min at pH 8.0, 25 °C. The reduced protein is designated R, and the native protein is N. An Arabic numeral corresponding to the number of intramolecular disulfide bonds followed by S denotes the various groupings of disulfide-bonded intermediates. des-[40−95] and des-[65−72] correspond to species that contain three native disulfide bonds but lack the 40−95 and the 65−72 disulfide bonds, respectively.

HPLC, the first run at room temperature, and the second at 50 °C.

RESULTS

Kinetic Trapping of Intermediates. As shown in Figure 1, the removal of the redox reagent from the regeneration mixture (Figure 1A) leads to a dramatic change in the distribution of 3S species (Figure 1B). As can be seen from Figure 1B, there are two major intermediates formed during the rearrangement process, suggesting the existence of two separate regeneration pathways of the 3S → 3S* type. This is confirmed by the identification of the disulfide pairings of the two species. The larger of the two peaks contains three-native disulfide bonds and lacks the 40−95 disulfide bond (des-[40−95]). The smaller peak also contains three native disulfide bonds but lacks the 65−72 disulfide bond (des-[65−72]). The use of the quantitative disulfide detection system in the peptide mapping procedure greatly increases the accuracy of the disulfide-pairing determination (*24*, *25*). Both des-[65−72] and des-[40−95] are pure species and are identical to those species identified during the reduction of RNase A (*21*, *22*).

Properties of des-[65−72] and des-[40−95]. Both species have nativelike CD spectra (*22*). Activity measurements of blocked des-[65−72] indicate that it is quite nativelike, retaining 75−80% of the activity of native RNase A (*22*, *26*). NMR structural studies (*26*, *27*) have shown that des-[65−72] is very similar to the native protein in all areas except for the loop region from residues 60−72, which contain the 65−72 disulfide bond. The activity of blocked des-[40−95] is only ∼5% of that of the native protein (*22*), and NMR studies of an analogue of this intermediate have been carried out (*28*). des-[40−95] also exhibits rather unusual elution properties on various chromatographic supports. As can be seen in Figure 1B, des-[40−95] elutes much later than either des-[65−72] or the other 3S species, eluting in the position of a 2S species. The elution position of des-[40−95] relative to the 1S and 2S species depends on the level of usage of the column, des-[40−95] being more strongly retained relative to other intermediates on a heavily used column (where it elutes in the 1S grouping). The recovery of des-[40−95], however, appears to be independent of the level of usage of the column and appears to be quantitative, based on re-injection and concentration vs peak area profiles. This unusual chromatographic behavior appears to be conformational since it can be eliminated by running the column at 50 °C but not at 40 °C. The melting point of the protein at neutral pH is expected to be between 40 and 50 °C (*22*, *29*).

Unfortunately, the unusual chromatographic behavior is not restricted to ion-exchange mechanisms. Use of the disposable 10DG desalting columns (which contain the polyacrylamide based resin P6DG) on regeneration samples containing known amounts of des-[40−95] indicate that des-[40−95] does not elute in the excluded volume of the column with the other regeneration intermediates. Examination of the included volume (the salt containing fraction) for the presence of des-[40−95] found that less than 25% (the precise amount depends on the absolute amount of des-[40−95] loaded) of the des-[40−95] placed onto the column was recovered. The recovery of des-[40−95] from Sephadex G-25 columns was ∼90%, similar to that for other intermediates including the native and fully reduced and blocked state. The implications that this behavior has on the previously published results (*13*, *14*) in which desalting was carried out on gel filtration columns will be presented in the Discussion.

New Procedures. The use of a strong-cation exchange column to fractionate the intermediates permits the desalting step to be carried out at the same time as the fractionation and analysis. This procedure was used successfully in our studies of the reductive pathways of RNase A (*22*). This eliminates the problems associated with the differential loss of protein using conventional gel-filtration procedures. Furthermore, the recovery of protein on the ion-exchange column is typically >99% as opposed to ∼95% under optimal conditions using gel filtration. In addition, since the ion-exchange fractionation results in multiple peaks, change in the elution position or area of any of the peaks can be monitored quantitatively, as a function of the amount of protein injected to detect preferential loss of any specific component. None was detected at temperatures below 30 °C, indicating that no significant preferential loss of intermediates occurs during the combined fractionation and desalting process. At temperatures above 40 °C, preferential loss of reduced and 1S species was observed.

Rate Constants. From the time dependence of the appearance of des-[65−72] and des-[40−95] and the disappearance of 3S, a determination can be made of the apparent rate constant of formation of the individual 3S* species from the 3S grouping. The measurement of this rate constant is complicated by several factors, most notably that, while no external redox reagent is present, some rearrangement, as reflected in formation of additional native protein and redistribution between groupings (not simply oxidation but reduction as well—compare the 4S region in Figure 1, panels A and B), is observed. This presumably arises from intermolecular interactions with a dimeric protein molecule as an intermediate or from trace amounts of DTT^{ox} or DTT^{red} that were not removed by desalting. This would result in a reduction in the amount of des-[65−72] and des-[40−95] measured, thereby leading to an underestimation of the rearrangement rate constants and an overestimation of the oxidation rate constants. In addition, as can be seen in Figure 1B, des-[65−72] and des-[40−95] are not baseline resolved from other species in the regeneration mixture. While these problems can be overcome, a more direct and accurate method for obtaining these rate constants would be to follow the formation of des-[65−72] and des-[40−95] during the regeneration process. This is beyond the scope of this paper, but data of that type are presented in the accompanying paper (*18*). At this point, we will settle for a less precise determination. The range of values for the rate constant corresponding to the 3S → des-[65−72] step is 0.001−0.003 min^{-1}, and for the 3S → des-[40−95] process the range is 0.006−0.018 min^{-1}.

The determination of the rate constants for oxidation of des-[65−72] and des-[40−95] by direct addition of DTT^{ox} to the rearrangement mixtures is subject to the same experimental difficulties discussed in the previous paragraph for the determination of the rearrangement rate constants. The range of values for oxidation of des-[65−72] is 0.05−0.3 M^{-1} min^{-1} and for des-[40−95] is 0.4−0.9 M^{-1} min^{-1}.

Fortunately, the values of the rate constants for the oxidation of des-[65−72] and des-[40−95] to native protein starting from pure isolated species can be measured much more precisely. These values, at pH 8.0 and 25 °C, are 0.139 ± 0.002 and 0.421 ± 0.011 M^{-1} min^{-1}, respectively. The errors are expressed at the 95% confidence limit. These experiments also indicate that the rates of oxidation of des-[65−72] and des-[40−95] have a linear dependence on the concentration of DTT^{ox} between 50 and 150 mM.

DISCUSSION

Comparison to Earlier Regeneration Studies. The results presented here have confirmed the accuracy of the conclusions of the earlier studies as to the identification of the type of rate-determining step by which RNase A regenerates (*13*, *14*). This is clearly demonstrated by our ability to use those conclusions to manipulate the redox conditions in such a way as to be able to isolate and identify specific disulfide-bonded intermediates that follow the rate-determining step in the regeneration process. These new results, however, raise some questions about some aspects of our earlier studies.

The effective rate constant for oxidation of des-[65−72] and des-[40−95] at the concentrations of DTT^{ox} used in earlier studies (40−200 mM) are not much larger than the rate constants for their formation (0.006−0.028 as compared to 0.001−0.003 min^{-1} and 0.017−0.084 as opposed to 0.006−0.018 min^{-1} for the des-[65−72] and des-[40−95] pathways, respectively). This indicates that both des-[65−72] and des-[40−95] should accumulate in the regeneration mixture. The existence of a peak corresponding to des-[40−95] can clearly be seen in the 2S region of the chromatogram of the regeneration mixture shown in Figure 1A. There was, however, no evidence of the presence of either species in earlier studies (*13*, *14*, *16*). As discussed by Rothwarf and Scheraga (*13*, *14*), the identification of the type of pathway at the level of groupings of intermediates is unaffected by small amounts of intermediates that interconvert slowly. Consequently, the presence of des-[65−72] and des-[40−95] does not alter any of the qualitative conclusions of the earlier analysis (*13*, *14*). However, the procedures used to carry out those studies were sufficiently sensitive that both nativelike three-disulfide species (especially des-[40−95]) should have been detected. The failure to detect des-[40−95] earlier can easily be explained by its quantitative removal by the 10DG desalting columns that were used in those experiments. In the experiments at low protein concentration and high DTT^{ox} concentration, desalting was carried out on a Sephadex G-25 column, and des-[40−95] should have been present. In fact it was. A small anomalous peak in the 1S region was observed in those experiments and was attributed to a modification of the protein from the blocking reagent when exposed to low pH (desalting on the Sephadex G-25 column in the earlier studies was carried out at pH 2.9) and was excluded from all analysis (*13*). We have since confirmed that those blocking and desalting conditions do not modify the protein (D. M. Rothwarf and H. A. Scheraga, unpublished results), and this peak was in all likelihood due to the presence of des-[40−95]. The correct way to treat des-[40−95] in the kinetic analysis used in our earlier work would be to exclude it, since it corresponds neither to the native protein nor to a species populated in the steady-state condition. This was done either directly by the desalting column or by assuming it to be an artifact peak.

As far as des-[65−72] is concerned, it is less obvious why it should have escaped notice. It has considerable activity, ∼75−80% (*22*, *26*), and it, therefore, seems likely that it would have been detected by activity measurements. However, the activity measurement of the groupings were made at regeneration times (60−120 min) closely following the establishment of the steady-state when the absolute concentration of intermediates was highest. Given the relatively small rate constant for the 3S → des-[65−72] step, it would appear likely that the concentration of des-[65−72] was simply too small to make a significant contribution. While one would expect that the accumulation of des-[65−72] should have been observable in either the experimentally determined equilibrium constants involving 3S or as a change in the distribution within the 3S grouping with time and redox conditions in those earlier studies, it was not. One reason for this failure to detect the existence of des-[65−72] is that the redox conditions used were not those under which high concentrations of des-[65−72] would accumulate. The optimum conditions for the accumulation of des-[65−72] are those that would favor 3S formation, i.e., a high redox ratio of DTT^{ox}/DTT^{red}, but with a low absolute concentration of DTT^{ox} so that the rate of oxidation of des-[65−72] would

be slow. Because, under these conditions, it was difficult to obtain high quality experimental data using the desalting procedures of the earlier study, they were not used. In addition, with the HPLC columns used in those earlier studies, des-[65−72] eluted in a crowded region of the 3S grouping; hence, changes in the chromatographic profile of the 3S group as a function of time or redox conditions were not apparent.

Again it should be reiterated that the presence of these two nativelike species, though unaccounted for in the previous studies, should have little affect on the conclusions or rate constants determined in those studies. This is demonstrated in the accompanying paper (*18*), which presents improved data sets that explicitly account for the presence of these two nativelike disulfide species.

The results presented here highlight the importance of sampling as much of the "redox space" as possible when trying to identify regeneration pathways. While in some ways the failure to detect these two nativelike three-disulfide species in the earlier studies can be viewed as fortuitous in that it made the kinetic analysis somewhat simpler, this is not true. Since it is specific pathways with identifiable intermediates that is the goal of these investigations, it is the deviations from the steady-state that are the most important to identify. The importance of the major methodological improvement made in this paper of elimination of a separate desalting step cannot be overstated. In addition to minimizing effects that result from selective loss of intermediates, it permits a wider range of redox conditions to be sampled accurately. This is because a high redox ratio (DTT^{ox}/DTT^{red}) and a low absolute concentration of DTT^{ox} requires very low concentrations of reduced protein (The starting concentration of reduced protein contributes significantly to the redox potential because formation of intramolecular disulfide bonds results in formation of DTT^{red}). This in turn requires large volumes of solution in order to obtain sufficient protein for accurate quantitation. This can also be achieved by removing the redox reagent from a steady-state mixture generated at high redox conditions and high concentrations of DTT^{ox} and restarting the regeneration process at a lower concentration of DTT^{ox} (or no DTT^{ox}, as we have done here).

des-[40−95] has been observed previously in the regeneration of RNase A when glutathione was used as the redox reagent (*13*, *30*). Its unusual chromatographic properties, especially its apparent ability to bind irreversibly to certain resins, makes the quantitation of any earlier studies suspect and, therefore, calls into question any conclusions about the significance of its role in the regeneration process with GSSG/GSH (*13*).

Comparison with Reduction Studies. Both of these intermediates have been observed in the reduction process, but it is important to remember that this does not necessarily mean that they are the same species structurally. Most of our detection and characterization occur on equilibrium conformations. However, the agreement between the rate constants for oxidation obtained directly from the addition of DTT^{ox} to the rearrangement mixture and from the reoxidation of the isolated reduction species suggest that they are structurally similar.

Multiple Pathways. The identification of two separate three-disulfide species following the rate-determining step would seem to suggest the existence of multiple regeneration pathways. However, the presence of populated intermediates does not necessarily mean that these intermediates are kinetically significant. They may accumulate to significant levels as a result of being more stable than the other three-disulfide species. Detailed kinetic studies are, therefore, required to elucidate the role of isolated intermediates in the regeneration process. The importance of such kinetic analyses has been demonstrated in the regeneration studies of BPTI, where it has been shown that a number of intermediates accumulate, but these intermediates are not directly involved in the subsequent folding (*5*, *8*).

Several possibilities exist for the role played in the regeneration process by des-[65−72] and des-[40−95]. One possibility is shown in the following scheme:

$$3S \rightarrow 3S^* \rightarrow N$$

$$3S \leftrightarrow \text{des-}[65-72]$$

$$3S \leftrightarrow \text{des-}[40-95]$$

where des-[65−72] and des-[40−95] do not go directly to native but correspond to dead end species, which accumulate only as a result of their thermodynamic stability not their kinetic significance, and 3S* represents a species that goes rapidly to native RNase A. In fact, des-[65−72] and des-[40−95] are much more stable than the other two nativelike three-disulfide species, des-[58−110] and des-[26−84], which have been produced and studied by recombinant methodologies (*29*). The measurement of the rate constants for oxidation of both des-[65−72] and des-[40−95] to native protein, which are larger than the comparable rates of oxidation of the 3S grouping (*14*, *18*), would seem to suggest that they are not dead-end species. However, those results do not exclude the possibility that des-[65−72] and des-[40−95] do not oxidize directly to native but rather are in equilibrium with other species which then oxidize to native. Fortunately, this possibility can be excluded by additional data obtained from reduction studies where the rates of rearrangement of des-[65−72] and des-[40−95] to other species have been measured (*22*). These results indicate that the rate of oxidation of des-[65−72] and des-[40−95] are too rapid to be accounted for by such a mechanism. Furthermore, the direct oxidation measurements carried out from pure species indicate that the rate of oxidation of both des-[65−72] and des-[40−95] exhibit a linear dependence on the concentration of DTT^{ox}. Therefore, both des-[65−72] and des-[40−95] oxidize directly to native, and consequently the two species must lie on separate regeneration pathways.

A second question is whether the two observed pathways (the des-[65−72] pathway and the des-[40−95] pathway) correspond to the only or even major regeneration pathways or whether another 3S* species exists that we did not observe but that would account for a significant amount of the native protein regenerated. Such a species would probably correspond to one of the other nativelike three-disulfide species, i.e., des-[26−84] or des-[58−110]. If some other 3S* species were kinetically significant, this would require that particular 3S* → N reactions be significantly faster than either des-[65−72] → N or des-[40−95] → N in order for it to make a significant contribution at its lower concentration.

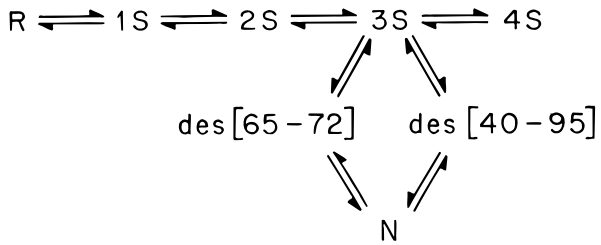

FIGURE 2: Regeneration model showing parallel pathways.

In addition, this rate would have to be significantly faster than that of the formation of 3S* or else it would lead to a situation where the regeneration rate would have a dependence on DTT^{ox}, i.e., the 3S + DTT^{ox} → N step would also have been found as the rate-determining step, not just 3S → 3S* (*14*).

From the rate constants for formation and oxidation of des-[65−72] and des-[40−95], these two species would seem within experimental error to be able to account for at least 50% (and possibly as high as 100%) of the native protein formed, but more precise measurements [which are presented in the accompanying paper (*18*)] are needed to resolve this issue unambiguously. Given that des-[40−95] forms faster and oxidizes to native faster than des-[65−72], it is clear that the des-[40−95] pathway is the major pathway.

These data are consistent with the regeneration model shown in Figure 2, although the possibility of additional pathways cannot be excluded. This model is also consistent with the results of Li et al. (*22*), which demonstrated that the reductive unfolding of RNase A proceeds through parallel pathways involving des-[65−72] and des-[40−95]. In the case of reduction, only those two pathways are significantly populated (*22*).

General Method. The results presented here represent the second step in the development of our general approach to the study of regeneration pathways. We have gone from knowledge of the type of rate-determining step to the identification of the intermediates that follow the rate-determining step using straightforward procedures which exploit the different redox properties inherent in the different types of rate-determining steps. Therefore, while our studies of the regeneration pathways of RNase A have resulted in our focusing on one type of rate-determining step (3S → 3S*), the ability to manipulate the redox conditions in such a way as to trap the species that follow the rate-determining step is applicable to essentially any of the possible types of rate-determining steps that might occur in other proteins.

We have completed the application of the second step of our procedure to RNase A and have identified two intermediates that follow the rate-determining steps in the regeneration process. The ultimate goal of these studies is the complete structural characterization of the regeneration pathways of RNase A and, in particular, the determination of the rate-determining structural transitions involved in all major pathways. To obtain this information, it is necessary to determine the species that directly precede the rate-determining step in each pathway. This then will be the third stage in our general procedure. The means by which we proceed from the results of the second stage to carry out the third stage are discussed in the accompanying paper (*18*).

ACKNOWLEDGMENT

We thank X. Xu for graciously providing much of the AEMTS used in these studies.

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BI972822N