Regeneration of Bovine Pancreatic Ribonuclease A. 1. Steady-State Distribution†

David M. Rothwarf and Harold A. Scheraga*

Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853-1301

Received October 20, 1992; Revised Manuscript Received January 5, 1993

ABSTRACT: The regeneration of bovine pancreatic ribonuclease A (RNase A) from the reduced to the native form with mixtures of oxidized and reduced dithiothreitol has been studied at 25 °C, pH 8.0, by using a variety of current experimental techniques, including quenching the regeneration reaction with 2-aminoethyl methanethiosulfonate, fractionation of intermediates by HPLC, and analysis by both UV and disulfide-specific detection systems. The disulfide-containing protein intermediates achieve a steady-state distribution after which the native protein regenerates at a rate comparable to the rates observed previously during the regeneration of RNase A with glutathione. Equilibrium contants at 25 °C, pH 8.0, for the interconversion of species containing different numbers of disulfide bonds are evaluated from the concentrations in the steady-state distribution. These equilibrium constants are compared with those obtained earlier when native RNase A is regenerated with glutathione. The observed equilibrium constants (with the dithiothreitol system) for the interconversions among all intermediates are very similar once statistical factors arising from the different numbers of disulfide-containing species in each grouping are taken into account. None of the disulfide-containing intermediates has any significant enzymatic activity, in agreement with earlier conclusions that these intermediates are considerably disordered. This is in sharp contrast to disulfide-containing intermediates populated during the regeneration of bovine pancreatic trypsin inhibitor, which have significant nativelike structure.

Since Anfinsen's (1973) landmark experiments first demonstrated that the amino acid sequence contains the necessary information to direct the folding of a protein, considerable effort has been devoted to determine detailed folding pathways. Through the characterization of intermediates populated along the folding pathways, it is hoped to be able to generate a set of empirical rules that will enable theoretical predictions to be made about folding pathways and protein structure. However, despite the accumulation of growing amounts of data, the problem is far from solved (Kim & Baldwin, 1990). Recent advances in genetic engineering have provided the means to exploit such knowledge, thereby adding a new urgency for the need to understand the process of in vitro protein folding.

Because the formation of disulfide bonds is governed by the thiol and disulfide concentrations of the redox couple used (under anaerobic conditions), it has represented the simplest approach to study the folding of proteins. By varying the concentrations of the small molecular weight thiol and disulfide components in solution, the distribution of intermediate disulfide forms as well as the rate of regeneration of native protein can be controlled in a predictable manner (Konishi et al., 1981, 1982a; Creighton & Goldenberg, 1984; Goldenberg, 1988). By quenching the disulfide-reshuffling process, folding intermediates can be isolated and studied. Even with these advantages, the problem remains nontrivial, and few pathways have been characterized completely.

The number of possible disulfide intermediates increases rapidly with the number of cystines in the protein, and success has been claimed with proteins having only three or fewer disulfide bonds (Creighton, 1985; Pace & Creighton, 1986; Weissman & Kim, 1991). The determination of a folding pathway for a 3-disulfide protein is already a difficult undertaking, there being 74 possible different internal disulfide-bonded intermediates excluding the reduced and native states and excluding the possibility of formation of mixed disulfides between free cysteines and the redox couple. A clearly defined pathway for a three-disulfide species has been proposed only in the case of bovine pancreatic trypsin inhibitor (BPTI)[1] (Creighton, 1985) and its homologous proteins (Hollecker & Creighton, 1983), although recent experimental evidence (Weissman & Kim, 1991, 1992) indicates that some aspects of the proposed pathway may be incorrect due to inadequacies of the quenching technique.

In the case of bovine pancreatic ribonuclease A (RNase A), which has 4 disulfides, there are 764 possible species (7193 if mixed disulfides with the redox reagent are included). The regeneration of RNase A by small molecular weight thiol redox couples has been studied in a number of laboratories (Hantgan et al., 1974; Ahmed et al., 1975; Creighton, 1977, 1979; Konishi et al., 1981, 1982a,b,c; Wearne & Creighton, 1988). The major common conclusion that can be drawn from these earlier studies is that the regeneration process of RNase A is significantly more complex than the regeneration of BPTI. During the regeneration of BPTI, only five well-populated disulfide-bonded intermediates accumulate (Weissman & Kim, 1991). Of these five, four have been shown to have attained the native conformation (Staley & Kim, 1992).

† This work was supported by Grant GM-14312 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 and from the Cornell Biotechnology Program. A preliminary summary of this work was published previously (Rothwarf & Scheraga, 1991).

* Author to whom correspondence should be addressed.

[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor; AEMTS, 2-aminoethyl methanethiosulfonate [$(NH_2)C_2H_5SSO_2CH_3$]; DDS, disulfide detection system; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent); DTT^{ox}, oxidized dithiothreitol; DTT^{red}, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; GdnSCN, guanidine thiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; NTB, 2-nitro-5-thiobenzoic acid; TEA, triethylamine; TEAA, triethylammonium acetate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/93/0432-2671$04.00/0

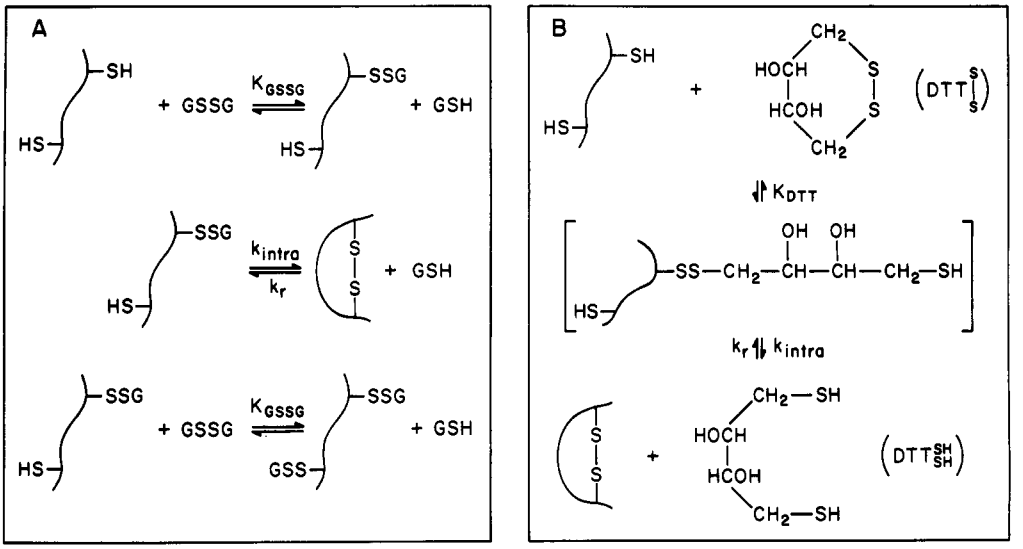

FIGURE 1: Formation of disulfide bonds by oxidation with (A) GSSG/GSH and (B) DTT^{ox}/DTT^{red}.

The rate-determining step in the formation of native BPTI is thought to be a rearrangement step in which a nativelike two-disulfide species rearranges to form another nativelike two-disulfide species (Weissman & Kim, 1991). All the structural folding has occurred by the time that the nativelike two-disulfides have formed, and, with the possible exception of one of the one-disulfide species, all the well-populated disulfide-bonded intermediates are not folding intermediates in a structural sense (Staley & Kim, 1992).

In contrast, during the regeneration of RNase A with a mixture of oxidized and reduced glutathiones (GSSG and GSH, respectively), the dominant interactions lead to disordered intermediates (Konishi & Scheraga, 1980b), indicating that the formation of disulfide bonds is crucial in directing the formation of subsequent native structure. Since the majority of native proteins (and presumably their folding intermediates) are significantly less stable than BPTI (or RNase A), it is unlikely that the formation of completely native structure in the absence of most of the disulfide bonds, as observed with BPTI, will be a common occurrence. It is more likely that the complex interplay between disulfide bond formation and the formation of native structure, as suggested in this and earlier studies of the regeneration of RNase A, will be the more general phenomenon. Hence, it seems that information gained through a detailed determination of the regeneration pathways of RNase A will be more generally applicable than the analogous information obtained from studies of BPTI.

Two different mechanisms for the regeneration of RNase A have been proposed: (1) one in which a single pathway exists with the rate-determining step in folding being the formation of the final disulfide bond (Creighton, 1988; Wearne & Creighton, 1988); (2) one in which multiple pathways are adopted with different rate-determining steps for each pathway, the relative amount of native protein generated on each pathway depending on the solution conditions (Konishi et al., 1982a; Scheraga et al., 1984, 1987).

Both of the mechanisms proposed previously have led to considerable controversy, as summarized by Goto (1991, 1992). For the most part, the controversy has centered upon the interpretation of experimental data using GSSG/GSH and DTT^{ox}/DTT^{red}, respectively, as the redox pair, and not on the quality of the data themselves. The two reagents react differently with protein thiols. When glutathione is used, formation of intramolecular protein disulfides proceeds through the formation of stable mixed disulfides between glutathione and the protein. In the regeneration process with DTT^{ox}, formation of stable mixed-disulfide bonds does not occur because of the rapid recyclization of the dithiothreitol involved in the mixed disulfide. These processes are shown schematically in Figure 1, and the implications of the differences in the interactions of the two types of reagents for the regeneration process are presented in detail in an accompanying paper (Rothwarf & Scheraga, 1993b).

The formation of stable mixed disulfides between the protein and glutathione results in a situation in which the regeneration rate and distribution of intermediates are complex functions of the ratio of the oxidized and reduced forms of the redox couple. Using the same techniques that had proved successful for BPTI, Creighton (1977, 1979) was unable to determine the regeneration pathways of RNase A. Konishi et al. (1981, 1982a), recognizing that the complexity of the problem required new experimental approaches, developed a method to account for the observed regeneration kinetics. They showed that the disulfide-bonded intermediates which were in rapid equilibrium with the reduced protein attained a preequilibrium state. They proposed that these intermediates were separated from the native protein by a high-energy barrier. The rate-determining step in the folding process is the crossing of that barrier. The distribution of intermediates in this preequilibrium state is a function of the concentrations of the redox couple, i.e., GSSG and GSH. By determination of the concentrations of intermediate species under a variety of different GSSG/GSH ratios, preequilibrium constants were determined. These constants were then employed in a kinetic scheme to determine which species immediately preceded the rate-determining step in folding. This method of analysis clearly accounted for the experimentally observed kinetic complexity.

The regeneration rate was shown to depend on two factors. The first was the concentration of the intermediate that precedes the rate-determining step. The second was the type of process involved. There are three types of rate-determining steps: oxidation, reduction, and internal rearrangement, as shown schematically in eq 1–3 where –SH, –SSG, and —S—S represent a free cysteine residue, a cysteine residue involved in a mixed-disulfide bond with GSH, and a cystine residue, respectively, in the protein. Step 3 could involve reshuffling to a different species, or it could involve some type of conformational change. Each step has a different dependence on the thiol–disulfide concentration. On the basis of

$$\text{RNase A}\begin{bmatrix}\text{—SH}\\ \text{—SH}\end{bmatrix} + \text{GSSG} \rightleftharpoons \text{RNase A}\begin{bmatrix}\text{—SH}\\ \text{—SSG}\end{bmatrix} + \text{GSH} \quad (1)$$

$$\text{RNase A}\begin{bmatrix}\text{—SH}\\ \text{—SSG}\end{bmatrix} \rightleftharpoons \text{RNase A}\begin{bmatrix}\text{—S}\\ \text{—S}\end{bmatrix} + \text{GSH} \quad (2)$$

$$\text{RNase A}\begin{bmatrix}\text{—S}\\ \text{—S}\end{bmatrix}, \text{—SSG}, \text{—SH} \rightleftharpoons \text{RNase A}\begin{bmatrix}\text{—S}\\ \text{—S}\end{bmatrix}, \text{—SSG}, \text{—SH} \quad (3)$$

these types of considerations, six major rate-determinining steps were found, including those involving one- and two-disulfide species (Konishi et al., 1982a).

The validity of the preequilibrium analysis, the existence of multiple regeneration pathways, and the surprising result that a one-disulfide species was involved in a rate-determining step were the subject of considerable controversy and thought to be in conflict with the more traditional conclusions of Creighton (Creighton, 1985, 1986; Wearne & Creighton, 1988). Creighton (1980) observed that, under a single set of nonoptimal folding conditions, a nativelike three-disulfide species with significant activity accumulated, suggesting to him that the rate-determining step in the regeneration process was the formation of the final disulfide bond. No evidence was provided to support the kinetic significance of this three-disulfide intermediate, and it has in fact been shown experimentally not to accumulate under conditions in which the preequilibrium state is attained and to be able to account for less than 20% of the observed regeneration rate (D. M. Rothwarf and H. A. Scheraga, unpublished results).

Creighton (1979, 1988) further contended that one- and two-disulfide species could not be involved in the rate-determining step since his attempted regeneration of RNase A by DTT^{ox} produced only reduced protein, and one- and two- disulfide intermediates, which led him to the conclusion that only three-disulfide species were involved in the rate-determining step. In addition, on the basis of the apparent absence of stable intermediates upon reduction of RNase A with DTT^{red} in his experiments, Creighton (Creighton, 1988; Wearne & Creighton, 1988) concluded that the rate-determining step is the formation of the final disulfide bond. However, the existence of stable intermediates upon reduction of RNase A has been reported (Sperling et al., 1969; Rothwarf & Scheraga, 1991). Creighton's conclusion was based on an incorrect application of the principle of microscopic reversibility, and the assertion was made that the redox ratio or the type of redox reagent used does not affect the regeneration/reduction pathway (Wearne & Creighton, 1988). However, since all reduction and oxidation processes are bimolecular, involving a thiol or disulfide reagent, changes in the thiol/disulfide ratio can significantly alter the rates of oxidation and reduction and thereby change the pathway by which the protein regenerates. This issue will be developed in the three accompanying papers (Rothwarf & Scheraga, 1993a,b,c).

A final point of contention has centered on the validity of the existence of the preequilibrium state. Creighton (1985, 1986, 1988) has suggested that certain disulfide arrangements and cis/trans peptide bond isomers may be slow to arise and, therefore, a true preequilibrium condition does not exist among the intermediates. The data presented here and in an accompanying paper (Rothwarf & Scheraga, 1993b) will show that these potential difficulties and the existence of slow interconversions among some species do not invalidate the method of kinetic analysis developed by Konishi et al. (1982a) for determining the regeneration pathways of RNase A.

The data and their analysis, presented here and in the accompanying papers (Rothwarf & Scheraga, 1993a,b,c), will try to reconcile these two conflicting views. The major advance that these results represent derives primarily from the application of new experimental techniques that were not available when earlier studies were carried out. These include the use of HPLC to fractionate intermediates, use of a very fast-reacting reversible blocking agent which avoids disulfide bond reshuffling and further facilitates the fractionation of the intermediates, and regeneration with oxidized and reduced dithiothreitol which, by eliminating formation of mixed disulfides, greatly simplifies the analytical procedures and subsequent analysis. The data presented in this paper will show that the native enzyme can be produced by regeneration with DTT^{ox} and DTT^{red} at rates (following the establishment of the steady state) that are comparable to those obtained with GSSG and GSH. A steady-state distribution is attained by the disulfide-bonded protein intermediates during the regeneration of RNase A with DTT^{ox} and DTT^{red}, and the method of data analysis developed by Konishi et al. (1981, 1982a) will be applied in the accompanying paper (Rothwarf & Scheraga, 1993a). Comparison between regeneration with these two types of redox reagents will be presented, and probable errors in the experimental methods and interpretation of Creighton (1977, 1979) and Wearne and Creighton (1988) will be discussed here and in the three accompanying papers (Rothwarf & Scheraga, 1993a,b,c). Some discussion of the results and conclusions of Konishi and Scheraga (1980a,b) and Konishi et al. (1981, 1982a,b,c) will also be presented (Rothwarf & Scheraga, 1993a,b,c).

This paper and the three accompanying ones (Rothwarf & Scheraga, 1993a,b,c) are the first in a series in which we intend to determine the major regeneration pathways of RNase A explicitly, and provide relevant kinetic, thermodynamic, and structural details necessary for the complete characterization of these pathways.

MATERIALS AND METHODS

Materials. RNase A (type 1-A, Sigma) was purified by ion-exchange chromatography on a 2.5 × 45 cm S-Sepharose column (Pharmacia) equilibrated with 25 mM HEPES/1 mM EDTA, pH 8.0, at 25 °C using a linear gradient of 0–150 mM NaCl over 1000 min at a flow rate of 2 mL/min. DTT^{red} ultrapure was obtained from Boehringer-Mannheim. DTT^{ox} (Sigma) was purified by the method of Creighton (1977). AEMTS was synthesized as described by Bruice and Kenyon (1982). All other reagents were of the highest grade commercially available.

Reduction of RNase A. RNase A (~1–1.5 mM) was reduced with a 100-fold molar excess of DTT^{red} in a pH 8.0 buffer containing 100 mM Tris, 2 mM EDTA, and 4 M GdnSCN, for between 2.5 and 4 h at 25 °C; 0.5 mL of the solution of the reduced protein was then buffer-exchanged rapidly (~4 min) into 100 mM Tris/2 mM EDTA, pH 8.0, on an HR10/10 column packed with G25 superfine (Pharmacia) resin, with continuous sparging with argon at 25 °C. The delivery and detection system consisted of an LKB 2150 pump with Rheodyne 7125 injector and an ISCO UA-5 detector with type 9 optical unit and 280-nm filters. The solution of buffer-exchanged reduced protein was placed in a cuvette, and the absorbance at 275 nm was measured on a modified Cary 14 spectrophotometer (Denton et al., 1982) connected to a Prime 750 computer. The protein concentration was determined as described below. The maximum time

between the loading of the reduction mixture onto the column and initiation of the regeneration process was less than 15 min. Reduced protein treated in this manner exhibited no significant amount of oxidation (less than 0.3%) when analyzed as described later in this section.

Regeneration of RNase A. The regeneration process was initiated by addition of a solution of the reduced protein to a solution of 100 mM Tris/2 mM EDTA, pH 8.0, containing the desired amount of oxidized and reduced DTT under argon. The concentration of reduced protein ranged from 3.2 to 78 μM with the majority of experiments being carried out at 32 μM; the concentration of DTT^{ox} ranged from 40 to 200 mM, and the *starting* concentration of DTT^{red} varied from 0 to 2000 μM. 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.

Blocking. Aliquots were removed at selected time intervals, and the unreacted thiols were blocked by addition of AEMTS freshly dissolved in 100 mM Tris/2 mM EDTA, pH 8.0. The result of the blocking is the coupling of the $^{+}NH_3CH_2CH_2S$ moiety (cysteamine) to the protein thiols to form disulfide bonds. A 5–100-fold molar excess of AEMTS was used. The distribution of intermediates did not depend on the amount of excess blocking reagent. After 2 min, the sample was desalted as described below. Completeness of blocking was tested at several times during the regeneration process by rapidly desalting the protein (desalting takes less than 5 min), adding GdnSCN to a concentration of 4 M, and determining if any free thiol was present by use of Ellman's reagent. None was found.

Desalting. Several procedures were used to desalt the blocked intermediates. The majority of samples (those corresponding to RNase A concentrations of 32 μM or greater) were desalted on disposable 10DG columns (Bio-Rad) into 50 mM TEAA, pH 5.0, 25 °C. The recovery of protein was greater than 95%, on the basis of the UV absorbance at 275 nm. This level of recovery was observed for both the native and the fully-reduced AEMTS-blocked protein, indicating that there is no preferential loss of unfolded protein using this system, in contrast to losses that occur with sulfonated or carboxymethylated derivatives of RNase A (D. M. Rothwarf and H. A. Scheraga, unpublished results). This is due primarily to the fact that the AEMTS blocking reagent is positively charged in the pH range used. The net charge of the protein is increased, and this appears to increase solubility by compensating for the exposure of nonpolar groups. In addition, we have found that the use of TEA in the buffer system greatly increases the recovery of RNase A and its intermediates when desalting, probably as a result of the detergent-like nature of TEA. Desalting was base line; i.e., there were no detectable levels of either DTT^{ox} or DTT^{red} present by reversed-phase HPLC analysis (Rothwarf & Scheraga, 1992) when starting with 100 mM concentrations of these reagents. Desalted samples that were not used immediately were frozen at −80 °C until needed. There was no measurable change in the distribution of intermediates even after 2 years in storage.

In experiments with 3.2 μM RNase A, a Pharmacia HR16/50 column packed with G25 superfine was used for desalting. The samples were desalted into 100 mM acetic acid at 25 °C and lyophilized. The remainder of the chromatographic system was identical to that described for the buffer-exchange procedure for the reduced protein. Recovery was better than 90%. Desalting on the G25 column gave a distribution of intermediates that was indistinguishable from those of samples desalted on the 10DG columns when applied to the higher concentration, 32 μM, samples, even when diluted 10-fold. The ability to desalt intermediates of RNase A quantitatively is another major advantage of the AEMTS blocking reagent.

It is important to report that, at very high concentrations of both AEMTS (>100 mM) and DTT^{red} (>20 mM), we have observed some small levels of protein modification by AEMTS when the reaction mixture is subjected to low pH (below pH 3) during desalting. *No such modifications* were observed in any of our control experiments when desalting was carried out in 50 mM TEAA at pH 5.0 or above, even when using 100 mM AEMTS and 20 mM DTT^{red}.

Fractionation of Intermediates. Intermediates were fractionated at room temperature (~22 °C) on a Bakerbond CBX 4.8 × 5 mm column or on a Pharmacia HR5/5 column packed with Bakerbond CBX resin. Because of the use of a positively charged blocking group, the fractionation was superior to that obtained with negatively charged groups such as iodoacetate. We have observed that more-unfolded species of RNase A tend to be retained longer than more-compact species of the same charge on cation-exchange columns. Therefore, optimum resolution is obtained when the totally reduced protein has the highest positive charge. Salt gradients were applied in 25 mM HEPES buffer/1 mM EDTA, pH 7.0, as reported previously (Rothwarf & Scheraga, 1991). A Spectra-Physics SP8700 gradient system with an ISCO UA-5 type 9 optical unit and 280-nm filter was used. The detector of this system was interfaced to an a/d convertor, and the data were stored on a Prime 750 computer. There was no detectable change in the distribution of intermediates even when samples were buffer-exchanged into the pH 7 buffer and left for 10 h at room temperature. This indicates that disulfide interchange should not occur to any appreciable extent during the chromatographic procedure.

Disulfide Analysis. The disulfide detection system (DDS) of Thannhauser et al. (1985) was used to analyze the AEMTS-blocked intermediates, but with the addition of a Lee mini-inert solenoid valve interfaced to a home-built digital control box that permitted accurate splitting of the column effluent without loss of resolution. Seventy percent of the column effluent was directed toward an ISCO UA-5 detector with a type 9 optical unit and 280-nm filter to determine protein concentration, and the remaining 30% was directed to the disulfide analyzer at a back and forth switching speed of 2 cycles/s. All the data were digitized and stored on a Prime 750 computer. Ratios of the peak areas from the two chromatograms (Figure 2A,B) were computed, and a normalization constant was applied to give the best fit for the native protein having 4.0 disulfides and the totally reduced and blocked protein having 8.0 disulfides. Typical chromatograms are shown in Figure 2, and the number of disulfides in each individual peak in regions R, I, II, III, and N (as denoted in Figure 3) were within the values 8.0 ± 0.1, 7.0 ± 0.2, 6.0 ± 0.2, 5.0 ± 0.2, and 4.0 ± 0.1, respectively (error is calculated at the 95% confidence limit).

As an additional check on the assignment of the number of disulfides, all peaks that presumably contain an identical number of disulfides based on the ratio method were pooled. The blocked proteins were then reduced with DTT^{red} and run over a Waters Novapak C18 column using the disulfide analyzer for detection of sulfhydryl groups. Integrated areas of the cysteamine (arising from the blocking group) and reduced RNase A were used to determine the average number of mixed disulfides per protein molecule. In all cases, the expected number of blocking groups was found (within the

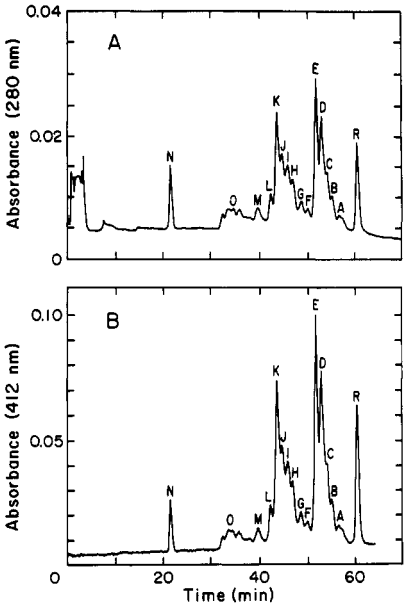

FIGURE 2: Chromatograms from a typical regeneration mixture: 100 mM DTT^{ox}, 32 μM RNase A, pH 8.0, 25 °C, 90 min. (A) Detected at 280 nm. (B) Detected by the DDS at 412 nm. The letters denote peaks that are well enough resolved to be integrated.

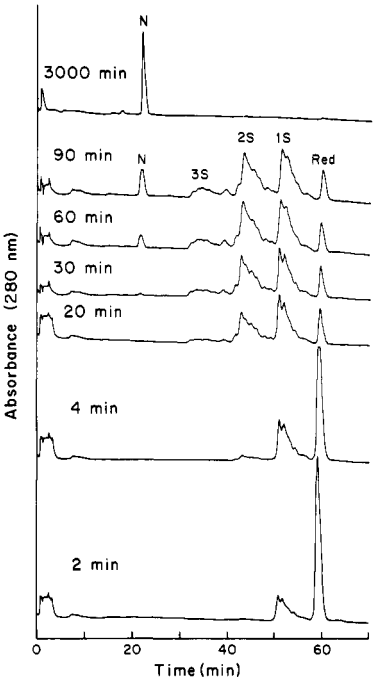

FIGURE 3: Series of chromatograms showing the appearance of intermediates with time. Regeneration conditions were 100 mM DTT^{ox}, 32 μM RNase A, pH 8.0, 25 °C.

error of the measurement, less than 0.1 blocking group at the 95% confidence limit). However, because of the error associated with any analytical method of this type, the possibility always exists that there is some significant overlap of the various disulfide species. An additional piece of information which supports the assignments made in Figure 2 is that, at different times during the regeneration, there was no appearance of a higher-order disulfide prior to the formation of the lower-order peaks; e.g., no peaks in the two-disulfide region were observed prior to the formation of the one-disulfide species (the peaks appear sequentially as would be expected by the number of disulfides that they contain). In addition, the entire family of peaks that correspond to a particular number of disulfide bonds tends to appear simultaneously. This is shown in Figure 3. As a result, a clear distinction can be made as to the number of intrachain disulfides present in each peak. These observations are in complete agreement with the data from the DDS. Moreover, observations on the distribution of intermediates under different redox conditions led to the same identification of the number of intramolecular disulfides within chromatographic peaks.

A more important point about the disulfide assignment data obtained from Figure 2 is that the relative ratios of peaks from 280-nm absorbance data from the ISCO detector are in good agreement with the data from the DDS (5–8%). This provides the basis for use of the 280-nm absorbance to determine the concentration of intermediates.

Protein Concentration. The concentration of reduced protein was determined by using 8600 M^{-1} cm^{-1} as the extinction coefficient at 275 nm in 100 mM Tris/2 mM EDTA, pH 8.0, and 25 °C. As will be explained in the first subsection under Results, the concentration of DTT^{red} depends on, and is determined by, the protein concentration. Consequently, any error in the measured concentration of reduced protein is carried over directly to the computed kinetic and equilibrium constants. We, therefore, used thiol analysis with Ellman's reagent, DTNB (Ellman, 1959), coupled with absorbance measurements of reduced RNase A at 275 nm to determine the extinction coefficient of the fully reduced protein. In this determination, a value of 13 900 M^{-1} cm^{-1} was used for the extinction coefficient of NTB at 412 nm (Thannhauser et al., 1984; Rothwarf & Scheraga, 1992). To test the accuracy of this method, it was first applied to reduced RNase A in 0.6% acetic acid. This led to an extinction coefficient of 8160 M^{-1} cm^{-1}. Konishi and Scheraga (1980a) had reported an extinction coefficient of 8100 ± 400 M^{-1} cm^{-1} for reduced RNase A in 0.6% acetic acid based on absorbance and nitrogen analysis. Given the excellent agreement between this result and that of Konishi and Scheraga (1980a) for reduced protein in 0.6% acetic acid, it is justified to use the same type of Ellman's analysis to determine the extinction coefficient (8600 M^{-1} cm^{-1}) of reduced RNase A in 100 mM Tris, pH 8.0. All values represent the average of three separate experiments. Within each experiment, four separate measurements were made with Ellman's reagent to minimize any errors.

All measurements described above and throughout this paper were made with Hamilton 1000 series gas-tight syringes or Finnpipette micropipettors which were calibrated before use. The standard deviation of the measurements was less than the 3% (99% confidence interval) in the extinction coefficient of NTB (Thannhauser et al., 1984). In determination of the extinction coefficient of the reduced protein, samples were tested to assure that oxidation or residual DTT^{red} did not affect the measurements. Reverse-phase HPLC analysis revealed no significant amount of residual DTT^{red}, and ion-exchange chromatography of reduced AEMTS-blocked protein indicated that no significant amount of oxidation occurred during the absorbance measurement.

Activity Measurements. Activity measurements were made by the method of Crook et al. (1960) as described by Konishi and Scheraga (1980a), with the modification that the substrate, cytidine cyclic monophosphate, was present in solution prior to the addition of a protein sample. All measurement were made at 25 °C and pH 5.0. Protein concentrations were determined by using the value of 9300 M^{-1} cm^{-1} at 275 nm for the extinction coefficient of native and partially regenerated protein (Konishi & Scheraga, 1980a).

Air Oxidation. Since the total amount of thiol should remain constant during the regeneration unless air oxidation occurred, all reaction mixtures were checked at the end of the regeneration experiment by Ellman's analysis. All data presented here had values within 5% of the expected one.

Hence, air oxidation is not a major consideration in the analysis of the data. An additional control was implemented in which reduced RNase A was placed under regeneration conditions in the absence of any redox reagent for up to 48 h. There was no significant change in the amount of thiol as indicated by Ellman's reagent. However, HPLC analysis of the same material (blocked with AEMTS) revealed that the blocked protein contained a significant amount of material that eluted earlier than the blocked fully reduced protein. This material eluted at the location expected for the blocked one-disulfide species and was present even, in a separate experiment, in the presence of large amounts (up to 100 mM) of DTT^{red}. The majority of this material probably corresponds to the known specific deamidation of Asn-67 (Thannhauser & Scheraga, 1985). [To minimize the effects of this deamidation process, no data beyond 10-h regeneration time were used to determine the apparent equilibrium constants. The amount of deamidation expected in 10 h at 25 °C and pH 8 is ~10% for the totally reduced protein and should be considerably less for the partially regenerated intermediates (Thannhauser & Scheraga, 1985; Wearne & Creighton, 1989). In addition, the actual amount of deamidated protein can be determined experimentally from the regeneration data by comparison of the native peak with the deamidated peak at the infinite time point. The deamidated material should also regenerate and should therefore appear in the HPLC chromatogram at infinite time (when all protein has been regenerated) as a peak which elutes slightly earlier than the native protein. As shown in the final 3000-min time point in Figure 3, the amount of deamidation (~5%), appearing just before the native peak, is significantly smaller than the amount of deamidation that would occur in the totally reduced protein.]

RESULTS

Steady-State Condition. A steady state was shown to exist for the regeneration intermediates of RNase A when GSSG and GSH were used as the redox couple (Konishi et al., 1982a). The data obtained here indicate that the DTT^{ox}/DTT^{red} system behaves similarly. Figure 4 shows representative progress curves (calculated with the rate constants of Table I) for the formation of different disulfide groupings. As the curves indicate, the intermediates achieve a steady-state condition after ~60–120 min. The actual time required for the attainment of the steady state depends on the starting concentrations of DTT^{ox} and DTT^{red} and the starting protein concentration. In the absence of air oxidation, the total concentration of thiol remains constant. Therefore, the starting concentration of protein contributes to the redox potential because formation of intramolecular disulfide bonds in the protein produces an equivalent amount of DTT^{red} (see Figure 1B). Once the intermediates achieve the steady-state condition, the only change in the distribution of intermediates results from the formation of native protein. The formation of native protein increases the concentration of DTT^{red}. This increase in DTT^{red} is partially offset by reequilibration of the intermediates with an accompanying conversion of DTT^{red} to DTT^{ox} so that the increase in DTT^{red} and the change in the distribution of intermediates are slow after the attainment of the steady state. This is clearly evident in Figures 4, 5, and 6.

Equilibrium Constants. The apparent equilibrium constants[2] between groups of species, distinguished by the number of intramolecular disulfide bonds, and the rate constants for the interconversion between groups, computed for the model shown in Figure 2 of Rothwarf and Scheraga (1993a) and discussed in that paper, are given in Table I.

[2] The apparent equilibrium constants determined from the concentrations of species measured after the attainment of the steady state will henceforth be referred to as equilibrium constants.

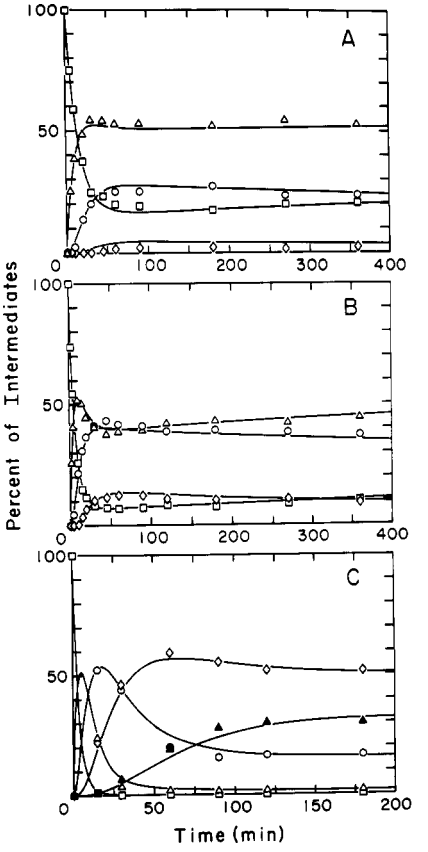

FIGURE 4: Representative curves showing the appearance of intermediates as a function of regeneration time at pH 8.0, 25 °C. (□) Reduced protein; (△) one-disulfides; (○) two-disulfides; (◇) three-disulfides; (▲) four-disulfides. Starting concentrations were (A) 40 mM DTT^{ox} and 32 μM RNase A, (B) 100 mM DTT^{ox} and 32 μM RNase A, and (C) 200 mM DTT^{ox} and 3.2 μM RNase A. Solid lines are theoretical curves drawn using the rate constants shown in Table I.

Table I: Equilibrium and Rate Constants[a] at 25 °C, pH 8.0

reaction	K_{eq}^{obs} [b] (×10⁴)	K_{eq}^{avg} [c] (×10⁴)	k_f^{obs} [d] (×10²) (min⁻¹ M⁻¹)	k_r^{obs} [e] (×10⁻²) (min⁻¹ M⁻¹)	K_{G-T} [f] (M)
R ⇄ 1S	31 ± 1 [g]	1.1	140 ± 10	4.5 ± 0.4	32
1S ⇄ 2S	5.7 ± 0.3	0.76	65 ± 9	11 ± 2	23
2S ⇄ 3S	1.7 ± 0.1	0.85	23 ± 3	11 ± 2	130
3S ⇄ 4S	0.33 ± 0.01	1.3	4.4 [h] ± 1.5	16 ± 3	710

[a] See Rothwarf and Scheraga (1993a) for the procedure to evaluate the rate constants. [b] This dimensionless equilibrium constant was calculated as an average over all experiments in which steady-state concentrations of the individual species were determined (see eq 4–7). [c] K_{eq}^{avg} is K_{eq}^{obs} corrected for statistical factors as described in the text. [d] k_f^{obs} is the observed rate constant for formation of a protein disulfide bond with DTT^{ox} for the reaction indicated here and in Figure 2 of Rothwarf and Scheraga (1993a). [e] k_r^{obs} is the observed rate constant for reduction of a protein disulfide bond with DTT^{red} for the reaction indicated here and in Figure 2 of Rothwarf and Scheraga (1993a). [f] Value calculated from eq 8 and 9 and similar equations for other species. The value obtained from direct experimental measurement is 229 ± 20 M (Rothwarf & Scheraga, 1992). [g] The error is calculated at the 95% confidence limit. [h] The rate-determining step is 3S → 3S*, for which the unimolecular rate constant is (9.8 ± 0.5) × 10^{-3} min^{-1} [see Rothwarf and Scheraga (1993a)].

The equilibrium constants reported in Table I pertain to the following equilibria:

$$R + DTT^{ox} \overset{K_A}{\rightleftarrows} 1S + DTT^{red} \quad (4)$$

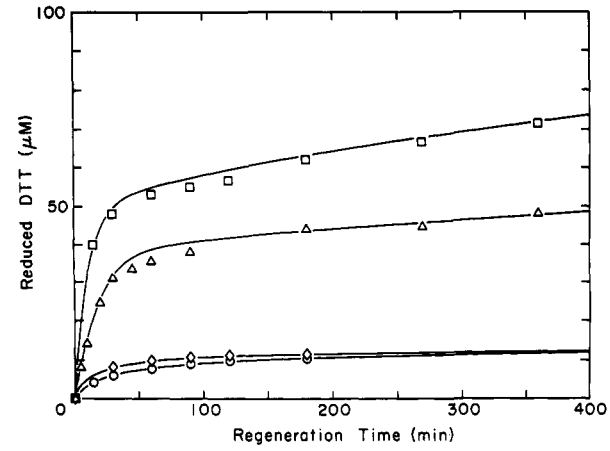

FIGURE 5: Curves showing the appearance of DTT^{red} as a function of regeneration time at pH 8.0, 25 °C. (△) 40 mM DTT^{ox}, 32 μM RNase A; (□) 100 mM DTT^{ox}, 32 μM RNase A; (○) 100 mM DTT^{ox}, 3.2 μM RNase A; (◇) 200 mM DTT^{ox}, 3.2 μM RNase A. Solid lines are theoretical curves drawn using the rate constants shown in Table I.

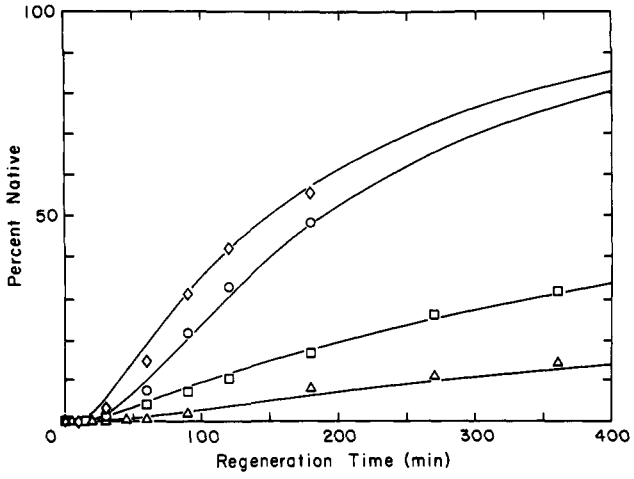

FIGURE 6: Plot of appearance of native protein as a function of regeneration time, at pH 8.0, 25 °C. Conditions are the same as for Figure 5.

$$1S + DTT^{ox} \overset{K_B}{\rightleftarrows} 2S + DTT^{red} \quad (5)$$

$$2S + DTT^{ox} \overset{K_C}{\rightleftarrows} 3S + DTT^{red} \quad (6)$$

$$3S + DTT^{ox} \overset{K_D}{\rightleftarrows} 4S + DTT^{red} \quad (7)$$

where 4S (which is enzymatically inactive; see below) does not include N. The equilibrium constants were calculated from the concentrations of species in the steady state. The solid curves in Figures 4–6 were computed using the rate constants reported in Table I.

Since the equilibrium constants are determined by using groups of intermediates, where each group contains a number of chemically distinct species, these equilibrium constants do not reflect the equilibrium constant between specific disulfide-containing species. While we cannot determine equilibrium constants between specific species with our current data, we can, however, determine an average value for the equilibrium constant between species within different groupings. For example, there are 28 possible 1-disulfide species and only 1 reduced species. Therefore, the average value of the equilibrium constant from eq 4 would be $K_A/28$. Average values of the other equilibrium constants were calculated in a similar manner and are shown in column 3 of Table I.

Further inspection of the data, and examination of the fine structure within each set of peaks that corresponds to a particular number of disulfide bonds, in most cases, reveals no change in distribution within each set of peaks as a function of time or redox conditions (Figure 3 serves as an example). However, under very strong oxidizing conditions and low protein concentration, we did observe a significant change in the distribution among the components of the one-disulfide species in some samples. A small peak persisted in the region of the chromatogram designated A in Figure 2 and gave the appearance of being the major component in the one-disulfide species. We believe that this peak could result from the use of 100 mM acetic acid during desalting and is the result of some modification of the three-disulfide or native protein by the blocking reagent. Since this peak accounts for at most ~3–4% of the total integrated area, it has a negligible effect on the results presented here. Values of the computed equilibrium constants that involve the one-disulfide species, K_A and K_B of eq 4 and 5, and Figure 2 of Rothwarf and Scheraga (1993a), do not contain data from the very high oxidizing condition experiments, and the presence of the peak was ignored in all further analysis. In general, this apparent impurity accounts for at most 4% of the total one-disulfide species used to determine K_A and K_B, and there was no indication of its appearance under less strongly oxidizing conditions.

Activity Measurements. Measurements of enzymatic activity showed that the peak assigned to native RNase A had 100% ± 3% (95% confidence limit) of native activity and that the rate of appearance of N in the chromatographic technique used to follow the regeneration correlates directly with the rate of regain of native activity. No other group in the chromatograms had any detectable activity within the 3% error under the conditions used. More sensitive assays on isolated intermediates under conditions more favorable to the stabilization of native structure, i.e., higher pH and lower temperature, will be implemented in future studies, when specific disulfide-bonded intermediates are identified. These activity measurements, however, are sufficient to demonstrate that the 3S species obtained in these regeneration experiments does *not* contain a significant amount of the nativelike enzymatically-active three-disulfide protein observed on reduction of native protein (Rothwarf & Scheraga, 1991).

DISCUSSION

Use of DTT^{ox}/DTT^{red}. The use of DTT^{ox}/DTT^{red} instead of GSSG/GSH has, as expected, greatly simplified the fractionation and identification of the intermediates that are populated during the regeneration of RNase A. The only complication in using DTT^{ox}/DTT^{red} rather than GSSG/GSH is the inability to maintain a constant redox potential during the course of the regeneration, and the smaller effective range of experimentally attainable redox potentials. This is a consequence of the relatively weak oxidizing power of DTT^{ox}/DTT^{red} which requires that a large ratio of $[DTT^{ox}]/[DTT^{red}]$ be used. As discussed above and shown in Figure 5, the formation of intramolecular protein disulfide bonds results in the formation of DTT^{red}. In order to maintain a constant redox potential, the starting concentration of DTT^{red} must be significantly greater than the thiol concentration of the reduced protein. Therefore, this places severe restrictions on the redox conditions which can be used and makes studies under constant redox potential experimentally impractical. In addition, regardless of the starting concentration of DTT^{red}, the concentration of reduced protein places a lower limit on the concentration of DTT^{red} that will be attained during the steady state, and hence an upper limit on the DTT^{ox}/DTT^{red} redox ratio.

Blocking with AEMTS. The reaction of thiosulfonates with a free thiol in solution under the conditions used to block the protein is known to be very rapid (Roberts et al., 1986), so that even thiols that are essentially buried should still be accessible by local or global fluctuations of the protein within the 2-min blocking time. Detailed kinetic studies of the rate of blocking of a variety of thiols in solution including protein thiols have been published for other members of the thiosulfonate family (Roberts et al., 1986). AEMTS reacts at a rate comparable to that observed with other members of the thiosulfonate family (Rothwarf & Scheraga, 1991). Protein thiols often appear to be inaccessible when blocking with other reagents, e.g., iodoacetate, which react at least 7 orders of magnitude slower than thiosulfonates (Rothwarf & Scheraga, 1991).

While it has been stated (Creighton, 1986) that irreversible blocking reagents, e.g., iodoacetate, are necessary to prevent the disulfides of proteins from reshuffling, this is incorrect. The criteria for a good blocking agent are that it block quickly, completely, and without modifying the protein at sites other than thiols. AEMTS is better able to meet these criteria than is iodoacetate, the traditionally advocated blocking reagent. In addition, there are advantages to a reversible blocking reagent since it permits the regeneration process to be restarted from isolated intermediates, an invaluable tool in the determination of regeneration pathways. Because of the speed and specificity of the blocking, thiosulfonates are the best blocking reagents yet available for studying the regeneration process.

Another reversible blocking method, low-pH quenching, was applied successfully in earlier regeneration studies of RNase A using GSSG and GSH (Konishi & Scheraga, 1980a,b; Konishi et al., 1981, 1982a) and in the recent regeneration studies of BPTI by Weissman and Kim (1991). However, pH quenching suffers from several experimental limitations. pH quenching does not stop thiol–disulfide exchange; it just slows it down by reducing the concentration of the reactive thiolate anion. This represents a serious limitation on the pH at which chromatographic separations and further characterization can be carried out. In addition, blocking with a reagent such as AEMTS provides a basis for separation of intermediates in a predictable manner. In the case of pH quenching, intermediates do not differ in charge unless they have formed mixed disulfides with the redox couple. While the reversed-phase HPLC separations of the regeneration intermediates by Weissman and Kim (1991) are impressive, separations are not predictable; i.e., very little information about the state of disulfide bonding is obtained from the elution position. In very complicated regeneration processes containing large numbers of chemically distinct species, it is impractical to utilize a separation that does not proceed in a predictable manner. In addition, reversed-phase HPLC separations are not well suited for larger proteins (Snyder et al., 1983; Geng & Regnier, 1984), such as RNase A. It is our belief that the blocking and fractionation methods presented in this paper are the most generally applicable, and represent an experimental framework suitable for use by those involved in the basic research problem of protein regeneration as well as those individuals and research groups interested in folding disulfide-containing proteins. While we have used AEMTS for our studies, other members of the thiosulfonate family may be better suited for regeneration studies of other proteins.

Distribution of Intermediates in the Steady State. It is important to note that the steady-state equilibrium constants presented in column 2 of Table I decrease by 2 orders of magnitude as the number of intramolecular protein disulfide bonds increases. However, this decrease does not appear to be due to conformational effects, but rather to originate from statistical factors, arising from the number of possible species within each disulfide grouping. As shown in column 3 of Table I, the value of K_{eq}^{avg}, which represents an average value for the equilibrium constant between individual species, is independent of the state of oxidation of the protein. Such an observation is in agreement with the observation of Konishi and Scheraga (1980b) that the intermediates are largely disordered.

Comparison to Other Regeneration Studies of RNase A Using DTT^{ox}/DTT^{red}. As mentioned in the introduction, it had previously been asserted that DTT^{ox}/DTT^{red} was incapable of regenerating RNase A (Creighton, 1977; Wearne & Creighton, 1988). However, the maximum rate of regeneration of native RNase A that we have observed here with DTT^{ox}/DTT^{red} at pH 8.0 and 25 °C, *after the attainment of the steady-state condition* [relaxation time of 169 min (as measured from those data shown in Figure 6 obtained *after* the attianment of the steady state), using 200 mM DTT^{ox} and 3.2 μM reduced RNase A], is similar to the maximum rate of regeneration obtained with GSSG/GSH by Konishi et al. (1982a) at 22 °C (relaxation time of 156 min) and the rate of regeneration under favorable but nonmaximal conditions with GSSG/GSH at 25 °C (relaxation time of 125 min) (Rothwarf & Scheraga, 1993c). This makes the failure of earlier investigators (Creighton, 1979; Wearne & Creighton, 1988) to observe the appearance of either the three-disulfide species or the native protein somewhat surprising, and is probably due to a number of factors discussed below.

Earlier studies using DTT^{ox}/DTT^{red} were conducted at pH 8.7 (Creighton, 1979; Wearne & Creighton, 1988) as opposed to pH 8.0 which we have used here. The reducing power of DTT^{red} increases with increasing pH, since the concentration of the reactive thiolate anion is elevated. Consequently, the concentration of the three-disulfide species populated in the steady state, under the strongest oxidizing conditions used in those earlier studies (60 mM DTT^{ox}, 30 μM reduced RNase A), would be low. A low concentration of the three-disulfide species, based on the regeneration model presented in Figure 2 of Rothwarf and Scheraga (1993a), corresponds to a low rate of regeneration of the native protein. However, we did observe a detectable rate of regeneration (~2% native protein/h after attainment of the steady state; see below) under those conditions (Rothwarf & Scheraga, 1991).

The electrophoretic method used in the earlier studies to determine the concentrations of the three-disulfide species and native protein was too insensitive to detect their formation. In contrast, using the chromatographic techniques presented here, where the native protein elutes in a sharp, well-resolved peak, we are able to determine the concentration of native protein quantitatively even when it corresponds to less than 0.1% of the total regeneration mixture.

A final contributing factor to the inability to detect the formation of native protein in earlier studies is the pronounced lag in the formation of this species (Figure 6), and the short regeneration times used [90 min was the longest regeneration time used by Wearne and Creighton (1988)]. However, there still would have been at least 2% native protein formed within that time period (Rothwarf & Scheraga, 1991).

Comparison of Equilibrium Constants. The equilibrium constants characterizing the preequilibrium condition observed by Konishi et al. (1981) can be compared to those obtained here. By using (i) the experimentally obtained preequilibrium constants of Konishi et al. (1981) and (ii) the equilibrium

constants obtained here for regeneration with DTT^{ox} and DTT^{red}, a test can be made of the consistency between the earlier data and those obtained here. For example, from K_A of eq 4 and a corresponding equation for the experiments of Konishi et al. (KOS) with GSSG/GSH, viz.

$$R + GSSG \overset{K_{KOS}}{\rightleftarrows} 1S + 2\,GSH \quad (8)$$

one can compute a value for the glutathione–dithiothreitol equilibrium constant K_{G-T}:

$$K_{G-T} = \frac{[DTT^{ox}][GSH]^2}{[DTT^{red}][GSSG]} = \frac{K_{KOS}}{K_A} \quad (9)$$

Similarly, K_{G-T} can be computed from K_B, K_C, and K_D, and corresponding data from Konishi et al. (1981). The last column of Table I shows the values of K_{G-T} determined by combining the preequilibrium constants of Konishi et al. (1981) with those presented here. These are compared with a value of K_{G-T} measured directly (Rothwarf & Scheraga, 1992). These data indicate that all of the values of K_{G-T} obtained from the regeneration data deviate by at most an order of magnitude from the value measured directly. Similar deviations of K_{G-T} have been observed between values determined during the regeneration of BPTI (Creighton & Goldenberg, 1984) and values measured directly (Chau & Nelson, 1991; Rothwarf & Scheraga, 1992). Such deviations could arise as a result of specific interactions between the redox reagents and the protein or could simply reflect experimental difficulties in determining regeneration preequilibrium constants using the experimental techniques available in the early 1980's. Therefore, considering the errors in the regeneration experiments with both redox couples, and the complexity of the stability of mixed disulfides between glutathione and the protein, we may conclude that both sets of experiments (those with the GSSG/GSH and DTT^{ox}/DTT^{red} systems, respectively) are compatible with each other.

CONCLUSION

Through the use of more recently available experimental techniques applied here to the regeneration of RNase A, the experimental results (using DTT^{ox}/DTT^{red}) differ from those obtained earlier (Creighton, 1979; Wearne & Creighton, 1988). The data obtained here are considered further in the accompanying papers (Rothwarf & Scheraga, 1993a,b,c).

ACKNOWLEDGMENT

We thank V. Gary Davenport for the construction of the digital control box used in the disulfide analysis and David R. Buckler for critical reading of the manuscript.

REFERENCES

Ahmed, A. K., Schaffer, S. W., & Wetlaufer, D. B. (1975) *J. Biol. Chem. 250*, 8477.
Anfinsen, C. B. (1973) *Science 181*, 223.
Bruice, T. W., & Kenyon, G. L. (1982) *J. Protein Chem. 1*, 47.
Chau, M. H., & Nelson, J. W. (1991) *FEBS Lett. 291*, 296.
Creighton, T. E. (1977) *J. Mol. Biol. 113*, 329.
Creighton, T. E. (1979) *J. Mol. Biol. 129*, 411.
Creighton, T. E. (1980) *FEBS Lett. 118*, 283.
Creighton, T. E. (1985) *J. Phys. Chem. 89*, 2452.
Creighton, T. E. (1986) *Methods Enzymol. 131*, 83.
Creighton, T. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5082.
Creighton, T. E., & Goldenberg, D. P. (1984) *J. Mol. Biol. 179*, 497.
Crook, E. M., Mathias, A. P., & Rabin, B. R. (1960) *Biochem. J. 74*, 234.
Denton, J. B., Konishi, Y., & Scheraga, H. A. (1982) *Biochemistry 21*, 5155.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.
Geng, X., & Regnier, F. E. (1984) *J. Chromatogr. 296*, 15.
Goldenberg, D. P. (1988) *Biochemistry 27*, 2481.
Goto, Y. (1991) *Kagaku (Kyoto) 46*, 47.
Goto, Y. (1992) *Tanpakushitsu Kakusan Koso 37*, 880.
Hantgan, R. R., Hammes, G. G., & Scheraga, H. A. (1974) *Biochemistry 13*, 3421.
Hollecker, M., & Creighton, T. E. (1983) *J. Mol. Biol. 168*, 409.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631.
Konishi, Y., & Scheraga, H. A. (1980a) *Biochemistry 19*, 1308.
Konishi, Y., & Scheraga, H. A. (1980b) *Biochemistry 19*, 1316.
Konishi, Y., Ooi, T., & Scheraga, H. A. (1981) *Biochemistry 20*, 3945.
Konishi, Y., Ooi, T., & Scheraga, H. A. (1982a) *Biochemistry 21*, 4734.
Konishi, Y., Ooi, T., & Scheraga, H. A. (1982b) *Biochemistry 21*, 4741.
Konishi, Y., Ooi, T., & Scheraga, H. A. (1982c) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5734.
Pace, C. N., & Creighton, T. E. (1986) *J. Mol. Biol. 188*, 477.
Roberts, D. D., Lewis, S. D., Ballou, D. P., Olson, S. T., & Shafer, J. A. (1986) *Biochemistry 25*, 5595.
Rothwarf, D. M., & Scheraga, H. A. (1991) *J. Am. Chem. Soc. 113*, 6293.
Rothwarf, D. M., & Scheraga, H. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7944.
Rothwarf, D. M., & Scheraga, H. A. (1993) *Biochemistry* (second of four papers in this issue).
Rothwarf, D. M., & Scheraga, H. A. (1993b) *Biochemistry* (third of four papers in this issue).
Rothwarf, D. M., & Scheraga, H. A. (1993c) *Biochemistry* (fourth of four papers in this issue).
Scheraga, H. A., Konishi, Y., & Ooi, T. (1984) *Adv. Biophys. 18*, 21.
Scheraga, H. A., Konishi, Y., Rothwarf, D. M., & Mui, P. W. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5740.
Snyder, L. R., Stadalius, M. A., & Quarry, M. A. (1983) *Anal. Chem. 55*, 1412A.
Sperling, R., Burstein, Y., & Steinberg, I. Z. (1969) *Biochemistry 8*, 3810.
Staley, J. P., & Kim, P. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1519.
Thannhauser, T. W., & Scheraga, H. A. (1985) *Biochemistry 24*, 7681.
Thannhauser, T. W., Konishi, Y., & Scheraga, H. A. (1984) *Anal. Biochem. 138*, 181.
Thannhauser, T. W., McWherter, C. A., & Scheraga, H. A. (1985) *Anal. Biochem. 149*, 322.
Wearne, S. J., & Creighton, T. E. (1988) *Proteins: Struct., Funct., Genet. 4*, 251.
Wearne, S. J., & Creighton, T. E. (1989) *Proteins: Struct., Funct., Genet. 5*, 8.
Weissman, J. S., & Kim, P. S. (1991) *Science 253*, 1386.
Weissman, J. S., & Kim, P. S. (1992) *Science 256*, 112.