Taborsky, G. (1959) *J. Biol. Chem. 234*, 2652.
Takahashi, S., & Ooi, T. (1976) *Bull. Inst. Chem. Res., Kyoto Univ. 54*, 141.
Takahashi, S., Kontani, T., Yoneda, M., & Ooi, T. (1977) *J. Biochem. (Tokyo) 82*, 1127.
Taniuchi, H. (1970) *J. Biol. Chem. 245*, 5459.
Walker, E. J., Ralston, G. B., & Darvey, I. G. (1978) *Biochem. J. 173*, 5.
Watkins, J. B., & Benz, F. W. (1978) *Science 199*, 1084.
White, F. H., Jr. (1967) *Methods Enzymol. 11*, 481.
White, F. H., Jr., Bello, J., Harker, D., & De Jarnette, E. (1961) *J. Biol. Chem. 236*, 1353.

Regeneration of Ribonuclease A from the Reduced Protein. 2. Conformational Analysis of the Intermediates by Nuclear Magnetic Resonance Spectroscopy†

Yasuo Konishi and Harold A. Scheraga*

ABSTRACT: Bovine pancreatic ribonuclease A was regenerated from the reduced protein by glutathione in the same way as described in the first paper of this series [Konishi, Y., & Scheraga, H. A. (1980) *Biochemistry* (preceding paper in this issue)]. The ^{1}H NMR spectrum of reduced ribonuclease A was typical of that of a disordered conformation. Since the intermediates in the early stages of the regeneration process exhibited a similar NMR spectrum in the aromatic region (but with less splitting of each resonance) as that of reduced ribonuclease A, the dominant conformations of the intermediates are more disordered. At all stages of the regeneration process, the resonance of each histidine residue [except His-12 C(2)] appeared as only two peaks, one corresponding to a disordered environment of the histidine residue and the other to that of native ribonuclease A. The resonances of the regenerated His-12 C(2) proton (which were also present in the spectrum of the reduced protein and in that of native ribonuclease A) disappeared in the spectrum of partially regenerated ribonuclease A, demonstrating a local conformational difference between regenerated and native ribonuclease A. It appears that the His-12 residue exists in two different environments in regenerated ribonuclease A (both being enzymatically active), and the slow interconversion between them broadens the resonance so that it disappears. The areas of the resonances of the regenerated His-48 C(2), His-105 C(2) and C(4), and His-119 C(2) protons in partially regenerated ribonuclease A were consistent with the degree of regeneration determined in the previous paper of this series. The area of the resonance of all His-C(2) protons corresponding to the disordered environment was also consistent with the degree of regeneration. Thus, the NMR data demonstrate that the dominant conformations of the intermediates are disordered.

The nuclear magnetic resonance (NMR)[1] technique has been used to study the folding of bovine pancreatic ribonuclease A (RNase A) (Matthews & Westmoreland, 1973; Benz & Roberts, 1975a,b). The resonances of His-12 C(2)-H, His-48 C(2)-H, His-105 C(2)-H and C(4)-H, and His-119 C(2)-H in native RNase A and of His C(2)-H in denatured RNase A can be resolved and have recently been reassigned (Bradbury & Teh, 1975; Markley, 1975a; Patel et al., 1975; Shindo et al., 1976). The chemical shifts and areas of these resonances provide two types of information. The chemical shift provides information as to whether a given residue is in the native, denatured, or some other state (Zaborsky & Millman, 1972; Blum et al., 1978), while the area of a resonance is proportional to the relative amount of a given conformation.

With this information, NMR studies have supported two models of the folding process. One is the two-state model proposed by Schellman (1955). Two-state behavior was observed in the thermal unfolding in D_2O at *high* pD (above pD 4.4); i.e., an identical decrease was observed in the areas of the four His C(2)-H resonances of the folded form with increasing temperature (Matthews & Westmoreland, 1973; Benz & Roberts, 1975a); similarly, no local unfolding of the environments of the aromatic residues was observed below the temperature of thermal unfolding (Lenstra et al., 1979). In the modification introduced by Chavez & Scheraga (1980a) into the unfolding pathway proposed by Burgess & Scheraga (1975), which pertains to unfolding near neutral pH in H_2O, intermediates are involved, with His-12 and -48 unfolding before His-105 and -119. The other model involves a stepwise unfolding. This behavior was observed in the equilibrium thermal unfolding in D_2O at *low* pD (below pD 3.4) (Matthews & Westmoreland, 1973; Benz & Roberts, 1975a) or in urea or guanidine hydrochloride unfolding (Benz & Roberts, 1975b); i.e., the environments of His-12 and His-119 *unfold* before the environments of His-48 and His-105. In a kinetic

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[1] Abbreviations used: NMR, nuclear magnetic resonance; RNase A, bovine pancreatic ribonuclease A; His C(2)-H and His C(4)-H, the C(2) and C(4) protons of histidine, respectively; 2'-CMP, cytidine 2'-monophosphoric acid; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt; UV, ultraviolet; ORD, optical rotatory dispersion; ΔOD_{287}, difference optical density at 287 nm; $\Delta[\alpha]_{436}$, optical rotation at 436 nm minus a constant value to bring it on scale; pD, pH of D_2O solution corrected with the relation pD = pH meter reading + 0.40 (Glasoe & Long, 1960); Tyr ϕ(2,6)-H, the ring protons at C(2) and C(6) of tyrosine; Tyr ϕ-(3,5)-H, the ring protons at C(3) and C(5) of tyrosine; Phe ϕ-H, the ring protons of phenylalanine; $(EA)_{rel}$, enzymatic activity of regenerated RNase A relative to that of native RNase A.

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study of folding in D_2O at low pD, Blum et al. (1978) also observed a multistate process, but reported that His-12 *folds* first. Recently, the ^{13}C NMR technique has been applied to the thermal unfolding of RNase A, demonstrating the presence of intermediate unfolding stages in D_2O particularly at *low* pD (Howarth, 1979).

The results of these NMR studies contradict those obtained by other techniques. In H_2O at low pH (< pH 3), studies of ultraviolet absorption (Hermans & Scheraga, 1961; Lumry et al., 1966), calorimetry (Tsong et al., 1970; Privalov et al., 1973), and binding of cytidine 2′-monophosphoric acid (Nall & Baldwin, 1977) indicate that the thermal unfolding is a two-state process. In H_2O at high pH (> pH 5), however, studies of IRC-50 column chromatography (Hirs, 1962), fluorescence (Young & Potts, 1963), circular dichroism (Simons et al., 1969), calorimetry (Tsong et al., 1970), laser Raman spectroscopy (Chen & Lord, 1976), hydrogen–deuterium exchange of tyrosine residues (Nakanishi & Tsuboi, 1978), nonspecific surface labeling (Matheson & Scheraga, 1979a), and temperature dependence of the enzymatic activity (Matheson & Scheraga, 1979b; Konishi & Scheraga, 1980) indicate the presence of conformational changes prior to the cooperative thermal unfolding. Conceivably, these contradictions might arise from solvent effects (D_2O vs. H_2O).

In order to circumvent this contradiction in the present study, the regeneration reaction was carried out in H_2O instead of D_2O and under the same conditions as in the study of the regeneration of the active site and of the UV and optical rotatory properties of the intermediates (Konishi & Scheraga, 1980). After the reaction was stopped, the partially regenerated RNase A was isolated and redissolved in D_2O solvent in order to measure the NMR spectrum. By carrying out the regeneration reaction in H_2O (even though the NMR measurements were made in D_2O), a direct comparison of the NMR results could be made with those of other techniques. In previous NMR studies, both regeneration and NMR measurements were carried out in D_2O. It should be noted that all of the NMR studies cited above were carried out on the *thermal* unfolding of ribonuclease A, with intact disulfide bonds. In contrast, the present study was carried out on the regeneration process, which involves formation and reshuffling of disulfide bonds. This work is also a kinetic study of the regeneration process (in H_2O) using the NMR technique. If any kinetic intermediates having ordered structure were to accumulate in the regeneration process, it might be possible to detect them with the NMR technique, as Blum et al. (1978) did in measurements in D_2O at low pD.

Experimental Section

Materials. The same materials were used as in the first paper of this series (Konishi & Scheraga, 1980). The reagents used for the NMR measurements were 99.7 atom % deuterium oxide, acetic-d_3 acid-d, and 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) from Aldrich Chemical Co., Inc., and 20% deuterium chloride in D_2O from Pharmacia Fine Chemicals. Urea for NMR measurements was dissolved in D_2O acidified to pD 2.4 with DCl to exchange all labile protons of urea for deuterium, allowed to stand for 1 h in order to remove any cyanate (Stark et al., 1960), and then lyophilized. The preparation of reduced and fully or partially regenerated RNase A is reported in the first paper of this series (Konishi & Scheraga, 1980).

NMR Measurements. A 250-MHz superconducting spectrometer system with a correlation technique (Dadok & Sprecher, 1974) was used to measure the ^1H NMR spectra of native, reduced, and partially regenerated RNase. The spectra are the results of 400 or 800 1.5- or 1.0-s scans in the low-field direction of a 1.5-kHz region downfield from the water peak. A time constant of 0.57 ms was used with a Princeton Applied Research phase detector amplifier, and the data were smoothed by using a 1.0-Hz digital filtering factor. The temperature of the protein solution was about 25 °C. An internal proton lock to the residual HDO peak was used to stabilize the field.

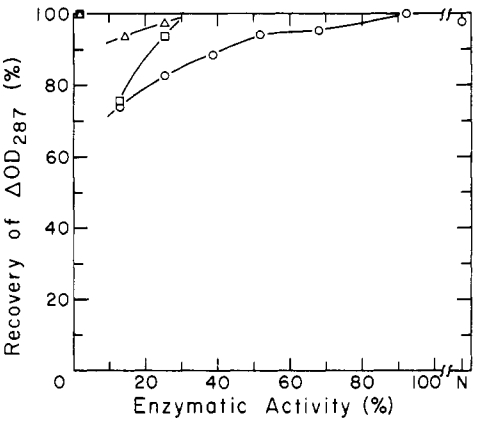

FIGURE 1: Percent recovery of the difference optical density at 287 nm after heating samples of different degrees of regeneration (expressed in terms of enzymatic activity) at 67 °C for 5 min (○), at 55 °C for 10 min (□), or at 55 °C for 5 min (△). Before the samples were heated, ΔOD was 100% in all cases. The concentrations of native and partially regenerated RNase A were 7.3×10^{-5} to 1.46×10^{-4} M in 0.6% acetic-d_3 acid-d–D_2O. In *all* cases, the *complete* enzymatic activity of the partially regenerated materials was recovered.

The protein solutions in 0.6% acetic-d_3 acid-d–D_2O were heated at 55–67 °C for 5–10 min to exchange the amide NH protons with deuterium. The solutions were then lyophilized and stored at −70 °C for 2–10 days. For the NMR measurements, the proteins were dissolved in 0.6% acetic-d_3 acid-d at a concentration of 2.9×10^{-3} M. The pD of the solution, obtained from the equation pD = pH meter reading + 0.40 (Glasoe & Long, 1960), was adjusted with 1 M DCl–D_2O or 1 M Tris base–D_2O. NMR spectra were taken with 0.4-mL protein solutions in 5-mm o.d. tubes. After recording an NMR spectrum, a small amount of DSS in D_2O was added to the protein solution to obtain the NMR spectrum of the internal standard. All chemical shifts are reported downfield from DSS in D_2O. The areas of the resonances were estimated by using a compensating polar planimeter (Keuffel & Esser Co.).

In order to check that heating in 0.6% acetic-d_3 acid-d–D_2O (to exchange the amide NH protons with deuterium) did not affect the conformation, we investigated the recovery of the protein conformation after heating by the recovery of the difference optical density at 287 nm (ΔOD_{287}) which reflects the recovery of the environments of tyrosine residues. The recovery was calculated by using the equation

$$\% \text{ recovery} = \left[1 - \frac{(\Delta OD_{287})_B - (\Delta OD_{287})_A}{(\Delta OD_{287})_{F-U}} \right] \times 100 \quad (1)$$

where $(\Delta OD_{287})_B$ and $(\Delta OD_{287})_A$ are ΔOD_{287} before and after heating, respectively, and $(\Delta OD_{287})_{F-U}$ is the difference of ΔOD_{287} between the thermally folded state and thermally unfolded state at room temperature (Konishi & Scheraga, 1980). The temperature at which the values of ΔOD_{287} were measured was 20 °C in all cases. The results, shown in Figure 1, indicate the conditions under which recovery of conformation can be kept above, say, 95%. Another check was made by measuring the enzymatic activity before and after heating. The enzymatic activities (measured at 20 °C) of native and

partially regenerated RNase A (enzymatic activity in the range of 10–90%) were recovered completely (within an experimental error of ±5%) after heating at 67 °C for 5 or 10 min. Thus, in the deuterium–hydrogen exchange experiments for NMR measurements, native or partially regenerated RNase A was heated under conditions in which both the values of ΔOD_{287} and the enzymatic activity recovered to at least 95%; hence, there was no irreversible loss (within experimental error) of conformation of the partially regenerated material in the exchange reaction.

Results

Restrictions on the pD in the NMR Measurement. There are three restrictions on the pD at which the NMR measurements of partially regenerated RNase A can be carried out. The first one arises from the solubilities of reduced and partially regenerated RNase, respectively. Both of these materials (with the latter having enzymatic activity in the range of 10–90%) showed poor solubility with an increase in pD above 4.9 at a protein concentration of 2.9×10^{-3} M. Since completely regenerated RNase A is soluble at this concentration, such poor solubility at high pD seems to be caused by the altered conformation of the protein. Many of the nonpolar residues are exposed to the solvent because of its disordered conformations; hence, the hydrophilicity of the protein is less than that of native RNase A. At low pD, the protein has a larger net charge, and the large electrostatic repulsion between the protein molecules solubilizes them. When the pD is increased, however, the net charge of the protein decreases gradually and becomes too small to solubilize the hydrophobic RNase A molecules in disordered conformations. Thus, the pD must be less than 4.9.

The second restriction arises from the possibility that the regenerated RNase A might be thermally unfolded (at room temperature) at low pD. The thermal transition temperature of RNase A depends on the pD of the solution in D_2O [similar to the dependence on pH in H_2O (Hermans & Scheraga, 1961)], and some of the regenerated molecules and the intermediates with ordered structures are thermally unfolded at low pD at room temperature. Such unfolding would lead to errors in the estimation of the amount of regenerated RNase A and intermediates with ordered structures. Unpublished data from experiments on thermal unfolding, obtained from the change of optical density at 287 nm, showed that native and partially regenerated RNase A are completely thermally folded at pD >3.8 and below 30 °C. Thus, the restriction that thermal unfolding be avoided requires that the pD of the solution must be above 3.8.

The third restriction is that the His C(2)-H resonances must be separable. NMR titration curves of the His C(2)-H resonances (Cohen & Shindo, 1975; Markley, 1975a; Benz & Roberts, 1975a; Westmoreland et al., 1975) showed that the resonances of His-12 C(2)-H, His-48 C(2)-H, and His-105 C(4)-H in the native conformation and of His C(2)-H in the disordered conformation are well separated from the other resonances in the range of pD from 3.8 to 4.9, which is the allowed range based on the solubility and thermal folding of partially regenerated RNase A. The resonances of His-105 C(2)-H and His-119 C(2)-H in the native conformation overlap with each other, and it is difficult to separate them in the range of pD from 3.8 to 4.9; the amount of regenerated environment of the His-105 residue, however, can be estimated from the area of the His-105 C(4)-H resonance in the regenerated conformation. Hence, subtraction of the area of this His-105 C(4)-H resonance from the summed areas of the His-105 C(2)-H and His-119 C(2)-H resonances gives the area of the His-119 C(2)-H resonance.

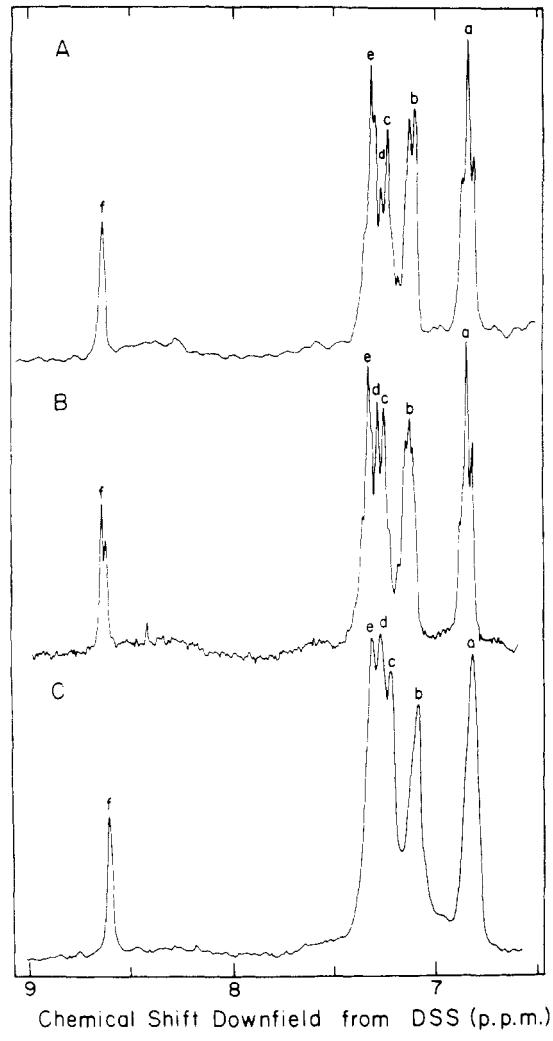

FIGURE 2: ^{1}H NMR spectra at 250 MHz (~25 °C) of reduced RNase A in 0.6% acetic-d_3 acid-d–D_2O and 8 M urea (pD 4.4) (A) and in 0.6% acetic-d_3 acid-d–D_2O (pD 4.4) (B) and of intermediates [at an early stage (6 min) of regeneration] in 0.6% acetic-d_3 acid-d–D_2O (pD 4.4). The concentration of protein was 2.9×10^{-5} M. The resonances are assigned as follows: (a) Tyr ϕ(3,5)-H; (b) Tyr ϕ(2,6)-H; (c–e) Phe ϕ-H and His C(4)-H; (f) His C(2)-H (Knowles et al., 1976).

Reduced RNase A. Figure 2A shows the NMR spectrum of reduced RNase A in 0.6% acetic-d_3 acid-d and 8 M urea–D_2O (pD 4.4, the low pD serving to prevent air oxidation), which was similar to that of native RNase A (not shown here) in the same solvent. The resonances were assigned by their areas and by the chemical shifts for amino acid residues incorporated into random-coil proteins (Knowles et al., 1976). The NMR spectrum of reduced RNase A in 0.6% acetic-d_3 acid-d–D_2O (pD 4.4) (Figure 2B) is similar to that in 8 M urea with a slight deviation, indicating that reduced RNase A in 0.6% acetic-d_3 acid-d–D_2O takes on a predominantly disordered but slightly different conformation from that in 8 M urea.

Intermediates at the Early Stage of Regeneration. Since the rate of reoxidation of reduced RNase A by reduced and oxidized glutathione is much faster than the rate of reshuffling of disulfide bonds (Hantgan et al., 1974; Creighton, 1977), it is possible to obtain reoxidized RNase A with a negligible amount of regeneration, i.e., at an early stage of regeneration. The NMR spectrum of such a partially regenerated RNase A (treated with glutathione for only 6 min) in 0.6% acetic-d_3

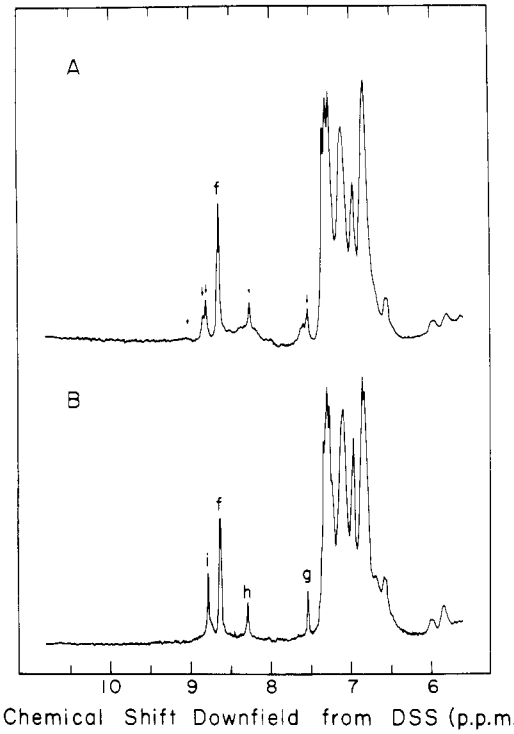

FIGURE 3: ^1H NMR spectra at 250 MHz (~25 °C) of partially regenerated RNase A (enzymatic activity = 44%) in (A) 0.6% acetic-d_3 acid-d–DCl–D_2O (pD 2.4) and in (B) 0.6% acetic-d_3 acid-d–D_2O (pD 4.6). The arrows in (A) show the chemical shifts of the resonances of His-12 C(2)-H, His-119 C(2)-H, His-105 C(2)-H, His-48 C(2)-H, and His-105 C(4)-H from low to high field in native RNase A at pD 2.4. The broad peaks around 7–9 ppm in (A) are the resonances of amide NH protons. f represents the His C(2)-H resonances from the disordered conformations. See the text for identification of peaks g–i.

acid-d–D_2O (Figure 2C), regeneration being inhibited during the NMR measurement by the low pD, shows single resonances for the Tyr ϕ(3,5)-H, Tyr ϕ(2,6)-H, and His C(2)-H protons. Each of these resonances was split into two to several resonances in the NMR spectrum of reduced RNase A (see Figure 2B). This indicates that the intermediates take on more disordered conformations than reduced RNase A. This is really interesting, because the reduced protein consists of an ensemble of conformations, all of which have the *same* covalent structure; in addition, the intermediates constitute a mixture of many kinds of species which have different combinations of disulfide bonds (Hantgan et al., 1974). Although it is expected that RNase A molecules with different disulfide bonds have different conformations, the single peak for each resonance indicates that the aromatic residues in these molecules are in the same or similar disordered environments.

Partially Regenerated RNase A. Figure 3A shows the NMR spectrum of partially regenerated RNase A (enzymatic activity = 44%) in 0.6% acetic-d_3 acid-d–DCl–D_2O at pD 2.4, where the protein would be partially thermally unfolded at room temperature. The chemical shifts of the resonances of the regenerated His-48 C(2)-H, His-105 C(2)-H, His-105 C(4)-H and His-119 C(2)-H were consistent with those of the native protein which are denoted by the arrows (Markley, 1975a). In addition, a broad peak was observed at the position of the resonance of native His-12 C(2)-H; it is indistinguishable from the resonances of the amide NH protons. The disappearance of the His-12 C(2)-H resonance does not mean that the environment of His-12 is unregenerated, because His-12 is in the active site of native RNase A and regeneration of His-12 is necessary for catalytic activity (Crestfield et al., 1963; Kenkare & Richards, 1966; Hofmann et al., 1970), which we observed for the partially regenerated RNase A (Konishi & Scheraga, 1980). Furthermore, the fact that the thermodynamic parameters for the initial velocity for the fully or partially regenerated RNase A (enzymatic activity in the range of 0.6–100%) are similar to those for native RNase A (Konishi & Scheraga, 1980) demonstrates that the active site and the environment of His-12 are regenerated in the regenerated molecule. Another possible explanation for the disappearance of the resonance is that the environment of the His-12 residue in the regenerated molecule might be distorted; this could shift the resonance so that it overlaps with other resonances. If this occurred, the enzymatic activity of the fully regenerated molecule would be different from that of native RNase A; the regenerated molecule which was purified by carboxymethylcellulose column chromatography, however, showed the same enzymatic activity as that of the native protein (Konishi & Scheraga, 1980). Thus, a distorted environment of His-12 cannot be the cause of the disappearance of this resonance. The explanation, which was given for the disappearance of the His-48 C(2)-H resonance in sodium chloride solution at pD >4.9 (Roberts et al., 1969; Meadows et al., 1969; Markley, 1975b), may apply here; viz., there may be at least two His-12 C(2)-H resonances corresponding to different environments of the His-12 residue, and the somewhat slow interconversion between them broadens the resonance and makes it disappear; presumably both conformations are enzymatically active.

Figure 3B shows the NMR spectrum of the partially regenerated RNase A (enzymatic activity = 44%) in 0.6% acetic-d_3 acid-d–D_2O at higher pD (viz., 4.6). The protein, which was thermally folded at this pD, showed only two resonances for each His C(2)-H except for His-12 C(2)-H, i.e., one (peak f) is for the disordered environments of four histidine residues and the other is for the regenerated environments of each histidine residue. This demonstrates that local regeneration of the environments of histidine residues occurs with only two conformations being detected or that the amounts of intermediates with ordered structures are too small to detect with the NMR technique. Thus, the total area of the disordered His C(2)-H resonance and the regenerated resonances of His-12 C(2)-H, His-48 C(2)-H, His-105 C-(2)-H, and His-119 C(2)-H should correspond to that of four protons. Since the His-12 residue is in the active site, we can reasonably assume that the amount of regeneration of the environment of the His-12 residue is the same as the regain of enzymatic activity; this provides a measure of the amount of His-12 regenerated.

The fraction of regenerated or unregenerated environments of histidine residues was calculated with eq 2–6 on a scale of 0.0–1.0.

$$\text{regenerated fraction of environment of His-12 residue} = (EA)_{rel} \quad (2)$$

$$\text{regenerated fraction of environment of His-48 residue} = [\text{His-48 C(2)-H}]_R/\{[\text{His C(2)-H}]_T/[4.0 - (EA)_{rel}]\} \quad (3)$$

$$\text{regenerated fraction of environment of His-105 residue} = [\text{His-105 C(4)-H}]_R/\{[\text{His C(2)-H}]_T/[4.0 - (EA)_{rel}]\} \quad (4)$$

$$\text{regenerated fraction of environment of His-119 residue} = \{\{[\text{His-105 C(2)-H}]_R + [\text{His-119 C(2)-H}]_R\} - [\text{His-105 C(4)-H}]_R\}/\{[\text{His C(2)-H}]_T/[4.0 - (EA)_{rel}]\} \quad (5)$$

$$\text{unregenerated fraction of environments of four histidine residues} = \{[\text{His C(2)-H}]_U/4.0\}/\{[\text{His C(2)-H}]_T/[4.0 - (EA)_{rel}]\} \quad (6)$$

where $(EA)_{rel}$ is the relative enzymatic activity of the sample

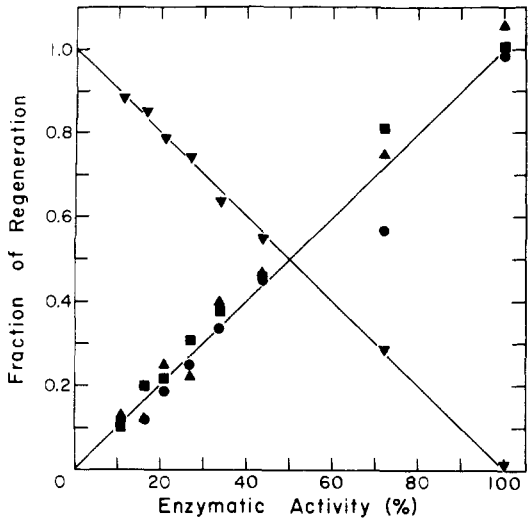

FIGURE 4: Fraction of regeneration of the environments of His-48 (●), His-105 (▲) and His-119 (■) and fraction of the environments of the four histidine residues in disordered conformations (▼) in partially regenerated RNase A are plotted against the enzymatic activity. The data at 100% enzymatic activity were obtained with native RNase A.

compared to that of native RNase A on a scale of 0.0–1.0 and $[\text{His C(2)-H}]_T$ is the summation of the areas of regenerated His-48, -105, and -119 C(2)-H resonances and the unregenerated His C(2)-H resonance; $[\text{His-48 C(2)-H}]_R$, $\{[\text{His-105 C(2)-H}]_R + [\text{His-119 C(2)-H}]_R\}$, $[\text{His-105 C(4)-H}]_R$, and $[\text{His C(2)-H}]_U$ are the areas of the resonances of peaks h, i, g, and f, respectively, of Figure 3B. They are plotted against the recovery of the enzymatic activity in Figure 4. The fraction of regeneration of the environment of each histidine residue is consistent with the recovery of enzymatic activity, and the fraction of the His C(2)-H residues in disordered conformations decreases correspondingly with the recovery of enzymatic activity, supporting the assumption of eq 2. Thus, the NMR data also support the conclusion that the dominant conformations of the intermediates are disordered.

Discussion

Effect of D_2O on Folding. The effect of D_2O on the native conformation of RNase A was studied by Roberts et al. (1969) using the NMR technique. They concluded that it was most unlikely that there was any conformational difference in RNase A between H_2O and D_2O which affects the environment of any of the histidine residues, because the pK values of the histidine residues obtained in D_2O were the same as those in H_2O and the same chemical shifts of the histidine residues were observed in H_2O and D_2O. Thus, our NMR data in D_2O can be compared directly with the other data in H_2O as shown in Figure 4.

D_2O, however, showed a different effect from that in H_2O in the glutathione regeneration of reduced RNase A. When the glutathione regeneration was performed in H_2O, at the high concentration of protein (2.9×10^{-3} M) required for the NMR measurements, a precipitate formed within 5 min, probably due to poor solubility or cross-linking of reoxidized and unregenerated RNase A at pH 8. At low concentration of protein (8.0×10^{-5} M), the protein did not precipitate, and the enzymatic activity increased up to 92% in the H_2O solvent system after 24 h. In the D_2O solvent system and at low concentration of protein (8.0×10^{-5} M), the regeneration reaction, which was followed by the regain of enzymatic activity (diluting out the turbidity mentioned below), was accelerated compared to that in the H_2O solvent system during the first stage of the reaction. During regeneration in D_2O, the solution slowly became turbid, and the apparent regeneration process slowed down markedly. Thus, D_2O (which accelerates regeneration but leads to turbidity) is not a good solvent for glutathione regeneration of reduced RNase A. This implies that D_2O might have a different effect from that of H_2O during protein folding. Perhaps this action of D_2O is due to its influence on hydrogen bonds (Hermans & Scheraga, 1959) and on hydrophobic interactions (Krescheck et al., 1965). For this reason, the regeneration was carried out in H_2O rather than in D_2O to obtain material for the NMR study.

Regeneration in H_2O. The possibility exists that a nucleation site for protein folding already appears in reduced RNase A and that the regeneration process is restricted by the nucleation site; i.e., the disulfide pairing may not be random initially. Even though the conformation of reduced RNase A has been shown to be close to that of a random coil (White et al., 1961; Tanford et al., 1966; Hantgan et al., 1974), the evidence that reduced RNase A has an enzymatic activity about 0.04% of that of native RNase A (Garel, 1978), that the equilibrium constant for the formation of the native conformation from the unfolded form is about 0.06 for reduced RNase A (Chavez & Scheraga, 1980b), and that the resonances in the NMR spectrum of the aromatic protons, especially His C(2)-H, of reduced RNase A are split (demonstrating heterogeneous environments for the aromatic residues) indicates some residual structure in reduced RNase A; this might arise because of the existence of a nucleation site. The NMR spectrum of the intermediates at an early stage of regeneration, however, indicated that the dominant conformations of the intermediates are more disordered than that of reduced RNase A. Thus, the residual structure in reduced RNase A seems to be disordered during reoxidation so that it cannot play a role in the regeneration process. The nucleation site, however, could reappear in the intermediates early in regeneration (Chavez & Scheraga, 1980a).

The NMR data also indicate that the conformation of regenerated RNase A, especially the environment of the His-12 residue, is slightly different from that of native RNase A. This might indicate isomerization of native RNase A, without loss of enzymatic activity. Indeed, many conformational isomers have been reported for native RNase A. For example, Craig et al. (1963) observed the existence of slowly interconvertible conformational isomers in a study of thin-film dialysis, Walker et al. (1978) found that the conformation of RNase A depended on the method of isolation, storage, and assay of the enzyme, Watkins & Benz (1978) obtained six fractions by Biorex 70 chromatography after incubation of native RNase A with mercaptoethanol, and Gutte (1978) obtained isomers in the air oxidation of reduced RNase A in the presence of nucleotides.

Conclusion

We summarize here the data which support the conclusion that the intermediates obtained in glutathione regeneration have disordered conformations. (1) The recovery of the enzymatic activity could be described by a single first-order rate constant in the kinetic study (Hantgan et al., 1974). (2) The re-formation of α-helical and β structure occurs at the same rate in the kinetic measurements made with circular dichroism (Schaffer, 1975). (3) The partially regenerated RNase A (enzymatic activity in the range of 10–90%) showed the same amounts of regeneration of the active site (as indicated by the enzymatic activity), of the environments of tyrosine residues (as indicated by the measured values of ΔOD_{287}), of the

backbone structure (as indicated by the measured values of $\Delta[\alpha]_{436}$) (Konishi & Scheraga, 1980), and of the environments of His-48, -105, and -119 residues as indicated by NMR measurements. (4) The temperature dependences of the enzymatic activity (i.e., the initial velocity) of fully or partially regenerated RNase A (enzymatic activity in the range of 0.6–100%) were the same as that of native RNase A (Konishi & Scheraga, 1980). (5) The thermodynamic parameters, T_m, $\Delta H°(T_m)$, and $\Delta S°(T_m)$, for changes in the environments of tyrosine residues or in the backbone structure, which showed a thermal transition in partially regenerated RNase A (enzymatic activity in the range of 20–90%), were the same as those of the native protein at pH 4.00 (Konishi & Scheraga, 1980).

This evidence, however, cannot exclude the possible presence of small amounts of intermediates having ordered structures. Indications for the presence of such intermediates are as follows. (1) Schaffer et al. (1975) observed a broad temperature optimum in the range of 30–37 °C for the rate and yield in the regeneration by glutathione. The increase in rate and yield at low temperature (below 30 °C) must be due to a positive Arrhenius activation energy for the regeneration reaction. The decrease in rate and yield at high temperature above 37 °C seems to be due to the thermal unfolding of ordered structures in the intermediate which changes conformation below the T_m of ~62 °C at neutral pH. The temperature optimum for the regeneration reaction was not influenced by adding phosphate, which interacts with the active site of the enzyme and greatly stabilizes the native conformation (Hermans & Scheraga, 1961; Barnard, 1964; Ginsburg & Carroll, 1965; von Hippel & Wong, 1965). This means that the active site is not regenerated in the intermediates. (2) About 17% of native conformation was observed for the intermediates in immunochemical experiments (Chavez & Scheraga, 1980a). (3) Creighton (1979) pointed out that the disulfide bonds are not distributed randomly (i.e., there are conformational restrictions for the formation of disulfide bonds) in species with three and four disulfide bonds, respectively. (4) The equilibrium constants for the thermal unfolding of partially regenerated RNase A showed small but possibly significant deviations at low temperature (below 50 °C) from those for the native protein [Figures 6 and 9 in Konishi & Scheraga (1980)], indicating the presence of ordered structure in the intermediates which are thermally unfolded at ~40 °C.

We conclude that the dominant conformations of the intermediates are disordered. Some intermediates, however, seem to have ordered structures which might play an important role, such as providing a nucleation site, in the regeneration process.

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References

Barnard, E. A. (1964) *J. Mol. Biol. 10*, 235.
Benz, F. W., & Roberts, G. C. K. (1975a) *J. Mol. Biol. 91*, 345.
Benz, F. W., & Roberts, G. C. K. (1975b) *J. Mol. Biol. 91*, 367.
Blum, A. D., Smallcombe, S. H., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 305.
Bradbury, J. H., & Teh, J. S. (1975) *J. Chem. Soc., Chem. Commun.*, 936.
Burgess, A. W., & Scheraga, H. A. (1975) *J. Theor. Biol. 53*, 403.
Chavez, L. G., Jr., & Scheraga, H. A. (1980a) *Biochemistry 19*, 996.
Chavez, L. G., Jr., & Scheraga, H. A. (1980b) *Biochemstry 19*, 1005.
Chen, M. C., & Lord, R. C. (1976) *Biochemistry 15*, 1889.
Cohen. J. S., & Shindo, H. (1975) *J. Biol. Chem. 250*, 8874.
Craig, L. C., King, T. P., & Crestfield, A. M. (1963) *Biopolymers 1*, 231.
Creighton, T. E. (1977) *J. Mol. Biol. 113*, 329.
Creighton, T. E. (1979) *J. Mol. Biol. 129*, 411.
Crestfield, A. M., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 2413.
Dadok, J., & Sprecher, R. F. (1974) *J. Magn. Reson. 13*, 243.
Garel, J.-R. (1978) *J. Mol. Biol. 118*, 331.
Ginsburg, A., & Carroll, W. R. (1965) *Biochemistry 4*, 2159.
Glasoe, P. K., & Long, F. A. (1960) *J. Phys. Chem. 64*, 188.
Gutte, B. (1978) *Eur. J. Biochem. 92*, 403.
Hantgan, R. R., Hammes, G. G., & Scheraga, H. A. (1974) *Biochemistry 13*, 3421.
Hermans, J., Jr., & Scheraga, H. A. (1959) *Biochim. Biophys. Acta 36*, 534.
Hermans, J., Jr., & Scheraga, H. A. (1961) *J. Am. Chem. Soc. 83*, 3283.
Hirs, C. H. W. (1962) *Brookhaven Symp. Biol. 15*, 154.
Hofmann, K., Visser, J. P., & Finn, F. M. (1970) *J. Am. Chem. Soc. 92*, 2900.
Howarth, O. W. (1979) *Biochim. Biophys. Acta 576*, 163.
Kenkare, U. W., & Richards, F. M. (1966) *J. Biol. Chem. 241*, 3197.
Knowles, P. F., Marsh, D., & Rattle, H. W. E. (1976) *Magnetic Resonance of Biomolecules*, pp 30–77, Wiley, New York.
Konishi, Y., & Scheraga, H. A. (1980) *Biochemistry* (preceding paper in this issue).
Krescheck, G. C., Scheider, H., & Scheraga, H. A. (1965) *J. Phys. Chem. 69*, 3132.
Lenstra, J. A., Bolscher, B. G. J. M., Stob, S., Beintema, J. J., & Kaptein, R. (1979) *Eur. J. Biochem. 98*, 385.
Lumry, R., Biltonen, R., & Brandts, J. F. (1966) *Biopolymers 4*, 917.
Markley, J. L. (1975a) *Biochemistry 14*, 3546.
Markley, J. L. (1975b) *Biochemistry 14*, 3554.
Matheson, R. R., Jr., & Scheraga, H. A. (1979a) *Biochemistry 18*, 2437.
Matheson, R. R., Jr., & Scheraga, H. A. (1979b) *Biochemistry 18*, 2446.
Matthews, C. R., & Westmoreland, D. G. (1973) *Ann. N.Y. Acad. Sci. 222*, 240.
Meadows, D. H., Roberts, G. C. K., & Jardetzky, O. (1969) *J. Mol. Biol. 45*, 491.
Nakanishi, M., & Tsuboi, M. (1978) *J. Am. Chem. Soc. 100*, 1273.
Nall, B. T., & Baldwin, R. L. (1977) *Biochemistry 16*, 3572.
Patel, D. J., Canuel, L. L., & Bovey, F. A. (1975) *Biopolymers 14*, 987.
Privalov, P. L., Tiktopulo, E. I., & Khechinashvili, N. N. (1973) *Int. J. Pept. Protein Res. 5*, 229.
Roberts, G. C. K., Meadows, D. H., & Jardetzky, O. (1969) *Biochemistry 8*, 2053.

Schaffer, S. W. (1975) *Int. J. Pept. Protein Res. 7*, 179.
Schaffer, S. W., Ahmed, A. K., & Wetlaufer, D. B. (1975) *J. Biol. Chem. 250*, 8483.
Schellman, J. A. (1955) *C. R. Trav. Lab. Carlsberg, Ser. Chim. 29*, 230.
Shindo, H., Hayes, M. B., & Cohen, J. S. (1976) *J. Biol. Chem. 251*, 2644.
Simons, E. R., Schneider, E. G., & Blout, E. R. (1969) *J. Biol. Chem. 244*, 4023.
Stark, G. R., Stein, W. H., & Moore, S. (1960) *J. Biol. Chem. 235*, 3177.
Tanford, C., Kawahara, K., & Lapanje, S. (1966) *J. Biol. Chem. 241*, 1921.
Tsong, T. Y., Hearn, R. P., Wrathall, D. P., & Sturtevant, J. M. (1970) *Biochemistry 9*, 2666.
von Hippel, P. H., & Wong, K.-Y. (1965) *J. Biol. Chem. 240*, 3909.
Walker, E. J., Ralston, G. B., & Darvey, I. G. (1978) *Biochem. J. 173*, 5.
Watkins, J. B., & Benz, F. W. (1978) *Science 199*, 1084.
Westmoreland, D. G., Matthews, C. R., Hays, M. B., & Cohen, J. S. (1975) *J. Biol. Chem. 250*, 7456.
White, F. H., Jr., Bello, J., Harker, D., & De Jarnette, E. (1961) *J. Biol. Chem. 236*, 1353.
Young, D. M., & Potts, J. T., Jr. (1963) *J. Biol. Chem. 238*, 1995.
Zaborsky, O. R., & Milliman, G. E. (1972) *Biochim. Biophys. Acta 271*, 274.

Activation of Bovine Factor XII (Hageman Factor) by Plasma Kallikrein†

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ABSTRACT: Bovine factor XII is a single-chain plasma protein that is involved in the early or contact phase of blood coagulation. In the reactions leading to fibrin formation, it is converted to factor XII_a, a serine protease composed of a heavy chain and a light chain held together by a disulfide bond(s). The activation of factor XII is catalyzed by plasma kallikrein which cleaves a single internal arginyl–valine peptide bond in the precursor protein. The activation reaction also requires the presence of a lipid component, such as sulfatide, ganglioside, or stearic acid. Kaolin, in the presence of high molecular weight kininogen, or dextran sulfate can provide a nonphysiological contact surface in this reaction. Other materials from bovine sources, including brain galactoceramide, sphingomyelin, phosphatidylcholine, cephalin, aorta proteoglycan, cornea keratan sulfate, vitreous humor hyaluronic acid, submaxillary mucin, and calf skin collagen, were inactive in the activation of factor XII by kallikrein. Factor XI_a and plasmin also activated factor XII in the presence of sulfatide but were only 40 and 20% as active as kallikrein, respectively. Other clotting enzymes, including factor IX_a, factor X_a, or thrombin, showed no activity in this reaction. The effect of sulfatide on reducing the clotting time of plasma suggests that some lipids, such as sulfatide, may be of importance in the initiation of the coagulation process, while others, such as phospholipids, play an important role in the intermediate phase of blood clotting.

Factor XII (Hageman factor)[1] is a glycoprotein (M_r 74 000) circulating in plasma in a zymogen form. It participates in the early or contact phase of blood coagulation, fibrinolysis, and kinin formation when these reactions are initiated by surface activation [see review by Kaplan (1978)]. Contact activation occurs on a negatively charged surface, such as glass, kaolin, Celite, or ellagic acid (Ratnoff & Rosenblum, 1958; Biggs et al., 1958; Nossel, 1964). Four plasma proteins, including factor XII, factor XI, prekallikrein, and high molecular weight kininogen (HMW kininogen),[2] are involved in these reactions. Present evidence suggests that factor XII is first converted to an active form and this initiates the various reactions leading to blood coagulation, clot lysis, and kinin formation. Recently, a factor XII dependent pathway for the renin–angiotensin system has also been described (Derkx et al., 1979).

The initiation of blood coagulation has been studied mainly with human preparations, and thus far three different types of mechanisms have been proposed: (1) activation of factor XII by limited proteolysis in a fluid phase by proteolytic enzymes such as kallikrein, plasmin, or factor XI_a, (2) activation of factor XII by limited proteolysis by the same enzymes on a negatively charged surface, and (3) activation of factor XII on a negatively charged surface leading to a conformational change in the protein and the formation of enzymatic activity. In the last mechanism, no fragmentation or cleavage of peptide bonds in factor XII is thought to occur.

Studies on the activation of factor XII were initially performed in a fluid phase using kallikrein, plasmin, or trypsin. In this system, the fragmentation of human factor XII into three peptide chains was reported (Kaplan & Austen, 1971; Cochrane et al., 1973; Revak et al., 1974). Later, it was found that the activation of human factor XII by limited proteolysis was far more efficient in the presence of a surface such as kaolin (Liu et al., 1977; Maier et al., 1977; Wiggins et al., 1977; Griffin, 1978). In addition, the surface activation was stimulated by the presence of HMW kininogen. Under these conditions, human factor XII was cleaved at one or two peptide bonds, resulting in the formation of factor XII_a containing three polypeptide chains with molecular weights of 40 000,

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[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

[2] Abbreviations used: HMW kininogen, high molecular weight kininogen; TAME, N^{α}-(*p*-tosyl)-L-arginine methyl ester.

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