# Glutathione-Facilitated Refolding of Reduced, Denatured Bovine Seminal Ribonuclease: Kinetics and Characterization of Products†

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ABSTRACT: Totally reduced and denatured seminal ribonuclease was regenerated using the glutathione redox system. The refolding kinetics of the enzyme were determined as a function of redox state, temperature from 14 to 43 °C, pH, and protein concentration. The maximal rate of regeneration occurred with $3 \times 10^{-3}$ M reduced glutathione, $6 \times 10^{-4}$ M oxidized glutathione, 24 to 30 °C, and pH 8.2. The products of the refolding process were characterized by Sephadex G-75, sodium dodecyl sulfate gel electrophoresis, enzymatic activity, circular dichroism, and amino acid analysis. The results indicate that the native dimeric form of the enzyme is not produced during refolding to any appreciable extent; rather, the major product is monomeric. The purified monomer exhibits twice the activity of the native enzyme toward yeast RNA. Its circular dichroism spectrum is different from the native enzyme and is quite similar to that of pancreatic ribonuclease A. Amino acid analyses showed that two glutathione molecules are bound to the monomer, suggesting that cysteine-31 and -32, which normally form the intermolecular disulfide bonds, are blocked.

Sela et al. (1957) originally developed the concept that the three-dimensional structure of a protein is determined by its amino acid sequence. There is now considerable evidence for this premise, although certain cofactors and environmental conditions also influence regeneration of native protein (Wetlaufer and Ristow, 1973; Anfinsen and Scheraga, 1975; Baldwin, 1975).

Refolding of the three-dimensional structure of oligomeric proteins has been of general interest since the original studies demonstrating reversible denaturation of hemoglobin (Anson and Mirsky, 1934). It is becoming increasingly apparent that for most oligomeric proteins the refolding of individual subunits precedes subunit reassociation (Bjork and Tanford, 1971; Freedman and Sela, 1966; Bornmann et al., 1974; Vimard et al., 1975; Tennenbaum-Bayer and Levitski, 1976). Chain refolding for aldolase and lactate dehydrogenase, the most extensively studied multimeric proteins, appears to be the rate-limiting step for regeneration while subunit association proceeds spontaneously at a rapid rate; however, this is not the case with all multimeric proteins. In this report we describe the regeneration of a protein, bovine seminal ribonuclease, in which the reassociation step is a major barrier in the acquisition of native structure.

Bovine seminal ribonuclease (seminal RNase) is a dimeric enzyme which contains two identical subunits connected by two intermolecular disulfide bonds (D'Alessio et al., 1975; Di Donato and D'Alessio, 1973). The subunit amino acid sequence of the RNase is strictly homologous with pancreatic RNase A; the major differences are the presence of four additional lysine residues, one additional proline, and two additional half-cystines located at positions 31 and 32 (Suzuki et al., 1976; Smyth et al., 1963). Since the subunits of seminal RNase and RNase A are similar, it was felt that a comparison could be made between the regeneration of the two enzymes. It also appeared attractive since it represented the first multimeric enzyme containing intermolecular disulfide bonds to be regenerated.

## Materials and Methods

*Materials.* Bovine seminal ribonuclease was prepared by the method of D'Alessio et al. (1972). Yeast RNA was purchased from Boehringer Mannheim (control no. 7305317), thoroughly dialyzed againat 0.1 M $NH_3 \cdot H_2O$ and lyophilized. 2-Mercaptoethanol and reduced and oxidized glutathione were purchased from Sigma Chemical Co. All electrophoresis reagents were products of Bio-Rad laboratories. 5′-UTP–hexane–agarose was purchased from P-L Biochemicals (lot no. 455131). Tris(hydroxymethyl)aminomethane and Ultra Pure urea were gifts of Chemzymes Inc.

*RNase Assay.* RNase activity was determined using a continuously recording method of Fletcher and Hash (1972) which was linear over the range employed. It was carried out at 30 °C in a thermostated Perkin-Elmer Hitachi 200 UV-vis spectrophotometer. The assay was typically run by adding 20 μL of enzyme to 3.0 mL of 75 μg/mL RNA solution buffered to pH 5.0 with 0.1 M sodium acetate. Activity is expressed as percent of native bovine seminal RNase activity. RNase concentration was determined spectrophotometrically at 278 nm ($\epsilon_{1cm}^{1\%} = 4.65$) after D'Alessio et al. (1972).

*Sulfhydryl Content.* Free sulfhydryls were assayed by the method of Ellman (1959) using $Nbs_2$ ($\epsilon_{max,412nm} = 1.36 \times 10^4$ $M^{-1}$ $cm^{-1}$).

*RNase Reduction.* Reduction of seminal RNase was carried out by a modification of the method of Anfinsen et al. (1961), in which 2 to 3 mg of native enzyme was incubated in 2.0 mL of 8.0 M urea, 0.01 M Tris,[1] 0.01 M EDTA, and a 500-fold excess of 2-mercaptoethanol, pH 8.5. The reduction was allowed to proceed for 3.5 h and was stopped by decreasing the pH to approximately 3.5 with 0.1 M acetic acid. The reduced, denatured enzyme was purified on a Sephadex G-25 column (60 × 1.5 cm) which was preequilibrated with 0.1 M acetic

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[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; $Nbs_2$, 5,5′-dithiobis(2-nitrobenzoic acid).

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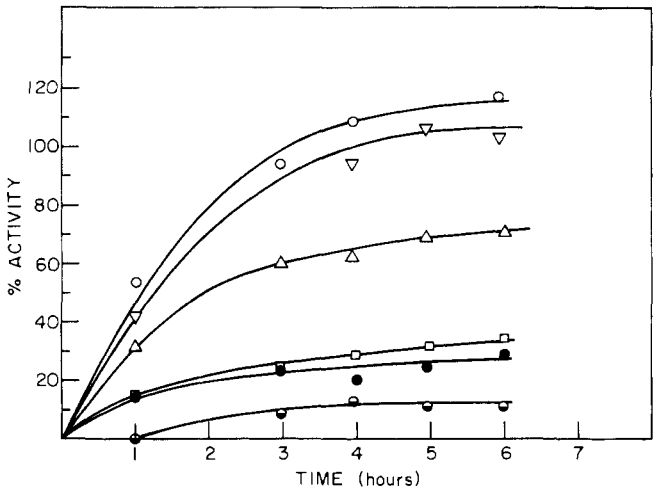


FIGURE 1: Effect of glutathione concentration and redox state on regeneration of seminal RNase. Seminal RNase (0.051 mg/mL) was regenerated in 0.07 M Tris-acetate, 0.7 mM EDTA, pH 8.2, buffer containing: (○) 3.0 mM GSH, 0.6 mM GSSG; (▽) 3.0 mM GSH, 0.3 mM GSSG; (△) 3.0 mM GSH, 3.0 mM GSSG; (□) 0.3 mM GSH, 3.0 mM GSSG; (●) 3.0 mM GSH, 10.0 mM GSSG; (⊖) 30.0 mM GSH, 3.0 mM GSSG at 30 °C.

acid. Following elution, the protein was either used immediately or was freeze-dried overnight and used the next day. Longer periods of storage resulted in loss of sulfhydryls and led to altered regeneration kinetics.

*Regeneration of Bovine Seminal RNase.* The regeneration was a modification of the method of Ahmed et al. (1975). Approximately 0.5 to 3.0 mg of reduced RNase was dissolved in 3.0 to 10.0 mL of distilled water, and the concentration was determined spectrophotometrically. The regeneration was started by pipetting 1.5 mL of the reduced protein into 3.5 mL of a 0.1 M Tris-acetate, 0.001 M EDTA (pH 8.2) buffer containing the desired concentration of reduced and oxidized glutathione and equilibrated at the desired temperature. Glutathione was always added just prior to the addition of protein to the reaction mixture. The regeneration kinetics were followed by removing 20-μL aliquots at the desired times for assay by the method described above.

*Preparative Reduction and Regeneration.* A typical preparation is described as follows: Five to fifteen milligrams of seminal RNase was reduced with a 500-fold excess of 2-mercaptoethanol at room temperature and purified on Sephadex G-25, in 0.1 M acetic acid. Tris and EDTA were added to the solution to bring their concentrations to 0.1 and 0.001 M, respectively. The pH was adjusted to 8.2 with 1.0 M NaOH. Regeneration began upon addition of glutathione. The concentration of protein in the regeneration medium varied from 0.1 to 0.30 mg/mL. The regeneration was allowed to proceed for approximately 17 h at 30 °C.

*Characterization of Products by Gel Filtration, Sodium Dodecyl Sulfate Electrophoresis, and Amino Acid Analysis.* The regeneration mixture was concentrated at room temperature by ultrafiltration with an Amicon UM-10 diaflow membrane prior to desalting on a Sephadex G-25 column (60 × 1.5 cm). The solution was further concentrated by ultrafiltration and was subsequently applied to an 80 × 1.5 cm Sephadex G-75 column preequilibrated with 0.1 M acetic acid. Elution from the column was determined by absorbance at 225 nm and enzymatic activity; void volume was established with blue dextran. The first protein peak was pooled and stored in either the freeze-dried state or at 4 °C. The second peak, following concentration by ultrafiltration, was routinely stored at 4 °C to avoid formation of protein aggregates during the freeze-drying step.

$NaDodSO_4$ electrophoresis was run on the native enzyme and both the regenerated monomer and dimer using a modification of the method of Weber and Osborn (1969). 2-Mercaptoethanol was eliminated from the denaturation mixtures to avoid reduction of the intermolecular disulfide linkages of native seminal RNase.

Amino acid analyses were performed according to Spackman (1967) with a Beckman Model 120 automatic analyzer. The seminal RNase monomer was purified by affinity chromatography on 5′-UTP-agarose (Smith et al., 1978), ultrafiltered several times with distilled $H_2O$ on an Amicon UM-10 diaflow membrane, and freeze-dried. Hydrolysis in 6 N HCl was carried out according to the procedure of Moore and Stein (1963).

*Circular Dichroism.* Measurements were made at room temperature on a Durrum-Jasco J-10 circular dichrometer, which was calibrated according to Cassim and Yang (1969). The monomer was prepared from the maximal glutathione redox mixture and purified by Sephadex G-75 gel filtration followed by affinity chromatography on 5′-UTP-agarose. Upon elution from the affinity column, the enzyme was concentrated on a UM-10 diaflow membrane and washed several times with distilled water and once with 0.05 M $KClO_4$, pH 3.9. The native enzyme was also subjected to affinity chromatography prior to analysis to assure homogeneity.

*Conversion of Regenerated Monomer to Dimer.* To 1.8 mL of 0.1 M Tris-acetate, pH 8.2, buffer containing glutathione of varying redox state was added 0.7 mL of 0.206 mg/mL of affinity pure monomer. Following 19 h of incubation at 30 °C a tenfold excess of iodoacetamide was added to stop the reaction. The products were characterized by $NaDodSO_4$ electrophoresis in the absence of 2-mercaptoethanol.

*Partial Reduction of Native Seminal RNase with Glutathione.* Native bovine seminal RNase at a concentration of 0.103 mg/mL was incubated in 0.1 M Tris-acetate, 1.0 mM EDTA, pH 8.2, buffer containing 3.0 mM GSH and 0.6 mM GSSG for 19 h at 30 °C. The modified enzyme was either first concentrated in 0.1 M acetic acid or 0.05 M Tris-Cl, 0.001 M EDTA (pH 7.2) on an Amicon UM-10 diaflow membrane or applied directly to an 85 × 1.5 cm Sephadex G-75 column equilibrated with 0.1 M acetic acid or 0.05 M Tris-Cl, 0.001 M EDTA (pH 7.2). Protein elution was followed by absorbance at 225 nm and enzyme activity. The protein peaks were pooled separately and characterized by $NaDodSO_4$ electrophoresis, performed in the absence of 2-mercaptoethanol.

## Results

Glutathione-facilitated regeneration of reduced, denatured bovine seminal RNase is characterized by an absolute dependence on the experimental conditions used in the regeneration studies. Figure 1 shows the effect of varying reduced and oxidized glutathione concentrations on the rate of enzymatic activity recovery. Both maximal rate and yield of enzymatic activity recovery were achieved at a concentration of 3.0 mM reduced glutathione (GSH) and 0.6 mM oxidized glutathione (GSSG). Changes in the redox state or the absolute concentration of reduced and oxidized glutathione resulted in decreases in reactivation rates. A similar dependence of RNase A regeneration on the redox state and glutathione concentration has been reported (Ahmed et al., 1975). However, one striking difference between the regeneration of the two proteins was apparent. Whereas glutathione-facilitated regeneration of RNase A yielded a product with about 90% native enzy-

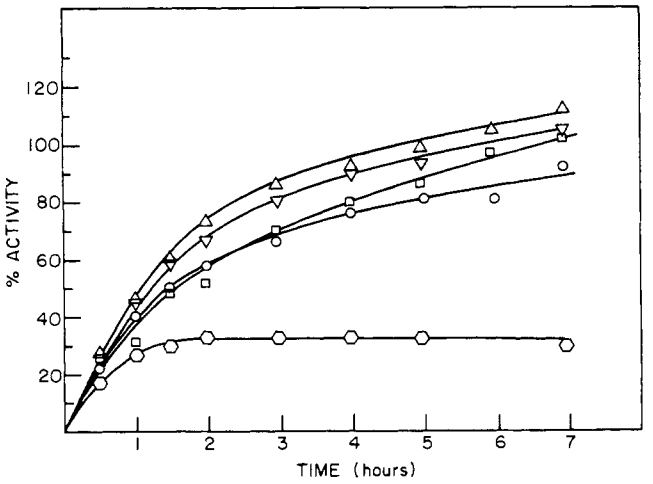


FIGURE 2: Effect of temperature on seminal RNase regeneration. Regenerations were carried out in a medium containing 0.091 mg/mL reduced seminal RNase, 3.0 mM GSH, and 0.6 mG GSSG and at a temperature of (△) 24; (▽) 30; (□) 14; (○) 36; and (◇) 42.7 °C.

matic activity, the seminal RNase product exhibited approximately 125% native activity. The reasons for this phenomenon will be discussed later.

The effect of temperature on the regeneration of seminal RNase under optimal glutathione conditions is seen in Figure 2. Very little effect of temperature was noted over a range of 14–30 °C. However, increases in temperature were characterized by decreases in both rate and extent of enzymatic activity recovery. When the regeneration was allowed to proceed to completion (24 h), the reaction products obtained from experiments performed at 14–30, 36, and 43 °C exhibited 110–125, 93, and 33% activity, respectively. This rather sharp temperature dependence above physiological temperature was not observed in RNase A refolding studies (Ahmed et al., 1975).

The regeneration was found to be highly dependent upon pH over a range of 6.8 to 8.0 (Figure 3). The results indicate that a group with a p$K \simeq 7.5$ controls the regeneration in this range. Above pH 8.0 the regeneration is independent of pH. This pattern is in agreement with the results from RNase A (Ahmed et al., 1975).

The effect of protein concentration on the regain of enzymatic activity was also examined (Figure 4). It was originally thought that the extent of enzymatic activity recovery would be highly dependent on protein concentration, since seminal RNase monomer was shown to exhibit an inherently greater specific activity toward yeast RNA than the dimer (see characterization of refolded seminal RNase monomer section). At higher protein concentration it was reasoned that dimer formation would be favored and that the recovery of enzymatic activity would be less than at lower protein concentrations where the monomeric form would be favored. However, recovery of activity was shown to be independent of protein concentration over a tenfold range under both optimal and nonoptimal glutathione concentration.

The kinetics of seminal RNase refolding under optimal conditions (pH 8.2, 30 °C, 3.0 mM GSH and 0.6 mM GSSG) were found to be quite complex; however, the data appeared to fit a pseudo-first-order process for the first 2 h of regeneration. The rate constant and $t_{1/2}$ calculated from the first-order plot are $3.35 \times 10^{-3}$ min$^{-1}$ and 200 min, respectively, based on recovery of active monomers which we will show subsequently has a specific activity of twice the native enzyme and is the major product of the regeneration.

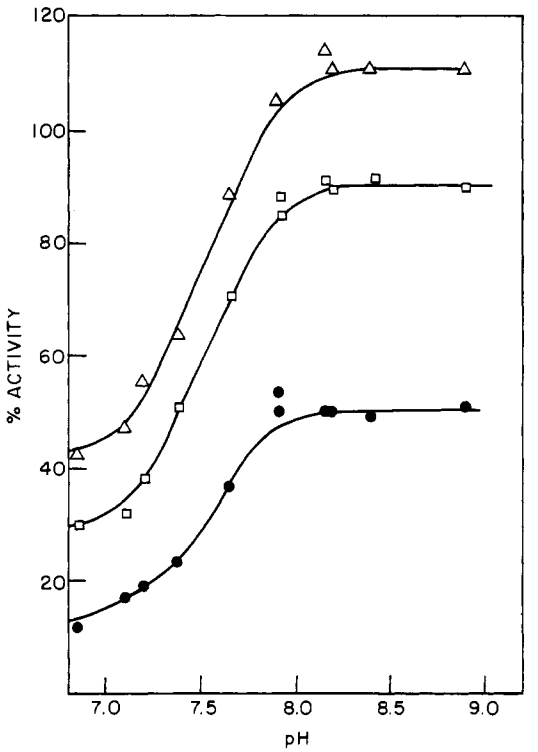


FIGURE 3: Effect of pH on regeneration. Regenerations were performed in Tris-acetate, 30 °C, buffer containing 3 mM GSH/0.6 mM GSSG. Protein concentration was either 0.12 or 0.17 mg/mL. Time points were determined at (●) 60; (□) 120; and (△) 180 min.

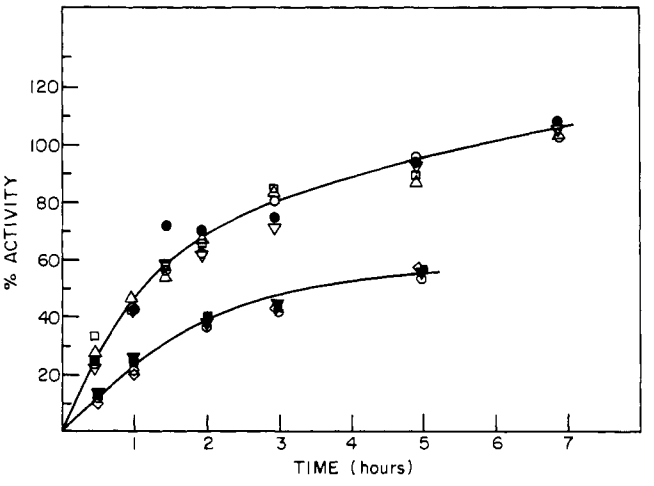


FIGURE 4: Effect of protein concentration on regeneration of seminal RNase under optimal and nonoptimal glutathione conditions. Regenerations were performed in Tris-acetate, pH 8.2, 30 °C buffer medium containing either 3.0 mM GSH/0.6 GSSG (upper curve) or 3.0 mM GSH/3.0 mM GSSG (lower curve) and a reduced seminal RNase concentration as follows: (◇) 0.293; (●) 0.140; (○) 0.137; (▽) 0.070; (■) 0.060; (○) 0.035; (△) 0.030; (▼) 0.029; (□) 0.018 mg/mL.

*Characterization of the Regeneration Products.* Figure 5 shows the Sephadex G-75 elution pattern of both native seminal RNase and regenerated seminal RNase prepared by incubating 0.1 to 0.3 mg/mL reduced protein in Tris buffer (pH 8.2) containing 3.0 mM GSH and 0.6 mM GSSG for 24 h at 30 °C. The major products of the reaction were found not to be native seminal RNase. Rather, the major peak eluted from the column in a position corresponding to that of seminal RNase monomer. Characterization of the pooled dimer peak by NaDodSO$_4$ electrophoresis indicated that the dimer peak

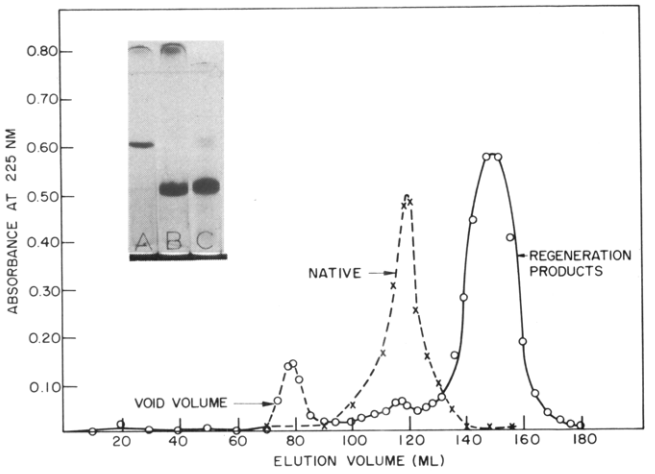


FIGURE 5: Sephadex G-75 elution pattern of both native and optimally regenerated seminal RNase. The regeneration was performed in 0.1 M Tris–acetate, 1.0 mM EDTA, pH 8.2, buffer containing 3.0 mM GSH/0.6 mM GSSG and 0.15 mg/mL of reduced seminal RNase. Following the 24-h incubation at 30 °C the protein was applied to an 89 × 1.5 cm Sephadex G-75 column and eluted with 0.1 M acetic acid. Inset: $NaDodSO_4$ gel electrophoresis of native seminal RNase and regeneration products. Electrophoresis of the native enzyme (A), Sephadex G-75 monomer peak (B), and Sephadex G-75 dimer (C) was carried out in the absence of 2-mercaptoethanol.

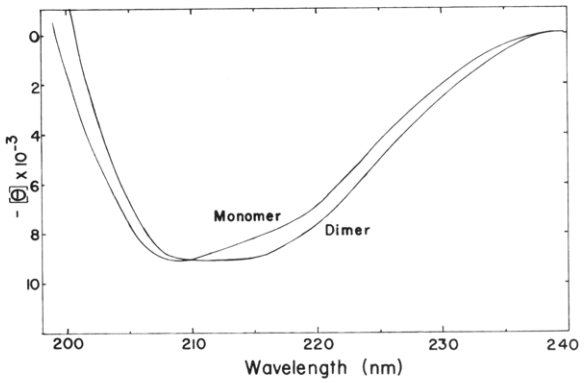


FIGURE 6: Circular dichroism of native seminal RNase and regenerated monomer. Circular dichroism spectra were carried out at pH 3.9 in 0.05 M $KClO_4$ on a Durrum-Jasco J-10 circular dichrometer. The concentrations of the monomer and the dimer were 0.774 and 0.643 mg/mL, respectively. [Θ] represents the mean residue molar ellipticity.

TABLE I: Partial Amino Acid Composition of Native Seminal Ribonuclease and the Refolded Monomer.[a]

| Amino acid | Native protein | Refolded monomer |
|---|---|---|
| Ala | 8 | 8.17 |
| Asp | 11 | 11.0 |
| Glu | 11 | 13.1 |
| Gly | 6 | 7.95 |
| Ile | 3 | 2.92 |
| Leu | 2 | 2.12 |
| Phe | 3 | 2.96 |

[a] Compositions are expressed as moles of residues per mole of subunits. Only the stable amino acids from the long column are included.

contained primarily noncovalent dimer, since most of the protein dissociated into monomers when exposed to $NaDodSO_4$ in the absence of reducing agents (Figure 5, inset). Two covalently linked dimeric species with slightly different mobilities were observed on $NaDodSO_4$ gel electrophoresis; however, it was impossible to establish if one of them corresponded to native seminal RNase, since both were present in the reaction mixture at an extremely low concentration.

Attempts were made to increase the yield of native dimer by increasing the concentration of GSSG in the regeneration medium. A twofold increase in GSSG concentration to 1.2 mM, while maintaining a GSH concentration of 3.0 mM, had no effect on the distribution of regeneration products. However, a further increase in GSSG concentration to 3.0 mM drastically reduced the amount of regenerated monomer. The Sephadex G-75 chromatographic pattern of the products from a 24-h regeneration in the presence of 3.0 mM GSH and 3.0 mM GSSG showed little or no monomer and essentially all dimer. Although these results suggest that a considerable amount of native dimer was possibly regenerated, this interpretation was not substantiated by $NaDodSO_4$ gel electrophoresis. The dimer peak from Sephadex G-75, which exhibited approximately 50% activity, was found to consist of largely noncovalent dimer.

*Characterization of Refolded Seminal RNase Monomer.* The monomer obtained from optimal regeneration conditions was purified initially on Sephadex G-75, which yielded a species with 150% activity. Subsequent purification using the affinity resin 5′-UTP–agarose (Smith et al., 1978) gave a preparation with 199 ± 7% activity, twice that of the native enzyme. The monomer appeared to be homogeneous upon electrophoresis with or without $NaDodSO_4$ (Reisfield et al., 1962; Weber and Osborn, 1969).

The pure monomer contained 0.005 to 0.018 free sulfhydryl per 14 500 daltons, which indicates that the sulfhydryls at residues 31 and 32 were not free. Amino acid analysis studies further showed that 2 mol of glutathione was incorporated into each monomer (Table I), which suggests that two mixed disulfides were formed with glutathione.

Circular dichroism spectra of native seminal RNase and regenerated seminal RNase monomer purified by affinity chromatography are shown in Figure 6. The major differences between the spectra of the two enzyme forms are the blue shift in both the trough and crossover for the monomeric form compared to the native enzyme. Although these changes are not large, it does suggest that the three-dimensional structure of the native enzyme is somewhat affected by its quaternary structure. Particularly noteworthy are the similarities between the spectra of RNase A and that of seminal RNase monomer (Schaffer, 1975). The data suggest that, during refolding, the monomer assumes a conformation similar to RNase A.

The regenerated monomeric form of the enzyme containing mixed disulfide bonds with glutathione proved to be extremely stable and resistant to covalent dimer formation. Attempts to induce significant dimerization of the glutathione-refolded monomer by air oxidation according to Ahmed et al. (1975) and glutathione redox mixtures of varying concentration all failed. Dimerization only occurred under two conditions, optimal glutathione ($3 \times 10^{-3}$ M GSH/$6 \times 10^{-4}$ M GSSG) and air oxidation. The yield of covalent dimer in both cases was very small (similar to Figure 5 inset), and it was impossible to determine if native enzyme had been formed. The presence of 0.5 mg/mL yeast RNA or 0.5 M inorganic phosphate failed to alter the amount of dimerization.

*Reduction of Native Seminal RNase with the Glutathione Redox System.* The regeneration studies suggest that the recovery of native, dimeric enzyme is prevented by the dimerization of the two subunits rather than the failure of the subunits to refold correctly. Although several factors could ac-

count for this observation, we examined the effect of glutathione on the native enzyme. We particularly were interested in determining the susceptibility of the intermolecular disulfide bonds to disulfide–sulfhydryl exchange with glutathione. This hypothesis was tested by treating native seminal RNase with conditions employed in the regeneration studies, namely, 3.0 mM GSH/0.6 mM GSSG for 19 h at 30 °C, pH 8.2. Although the results were complex, two definite points emerged. First, the native enzyme was partially reduced to yield a mixture of monomer and covalent dimer as shown by $NaDodSO_4$ gel electrophoresis. Activity measurements indicated that the products were 120–150% as active as the native enzyme. Second, it was found by $NaDodSO_4$ gel electrophoresis that partial reduction of the intermolecular disulfide bonds took place over a fairly broad range of glutathione concentration and redox state (GSH varied from 3.0 to 10.0 mM and GSSG from 0.6 to 10.0 mM). These data suggest that failure to regenerate the native dimer during glutathione-facilitated regeneration of seminal RNase is related to reduction of the two protein intermolecular disulfide bonds by glutathione.

## Discussion

The process of bovine seminal RNase activity regeneration in the presence of glutathione was found to exhibit many common features with regeneration of pancreatic RNase A. Characteristic of both processes was an absolute dependence on certain regeneration conditions. There was a pronounced effect of glutathione and temperature on the kinetics of refolding, with maximal regeneration occurring under reducing conditions and at moderate temperatures. The regeneration of both proteins was independent of protein concentration and proceeded to monomeric species which exhibit similar structural properties (Ahmed et al., 1975; Schaffer, 1975). However, three striking differences between the two processes were observed, which suggest slightly different folding pathways. First, the rate of seminal RNase monomer regeneration was slower and gave a lower yield than that of RNase A. RNase A regeneration was characterized by a $t_{1/2}$ of 75 min, while that of seminal RNase was 200 min. Second, the regeneration of RNase A yielded greater than 90% nativelike enzyme, while the seminal RNase products contained virtually no native enzyme and approximately 65% of the 200% active monomer (Ahmed et al., 1975). Third, both enzymes exhibited maximal rates of regeneration in the range 25–30 °C, and both processes were slower at lower temperatures. However, seminal RNase regeneration was affected more at higher temperatures than RNase A. The latter reached 75% activity at 42 °C after 240 min, while the former never rose above 33% of the *native* activity under similar conditions. Since seminal RNase contains two more half-cystine residues than RNase A, it is likely that the differences are partly due to the formation of different disulfide-containing intermediates during regeneration.

There is substantial evidence for both disulfide–sulfhydryl exchange and the formation of mixed disulfides between proteins and thiols (Modig, 1969; Harrap et al., 1973; De Lucia et al., 1975; Issacs and Binkley, 1977). The regenerated monomer contains essentially no free sulfhydryls but does possess two glutathione-mixed disulfides. The half-cystine residues involved in the formation of the mixed disulfides are assumed to be those located at positions 31 and 32. This conclusion is based upon the catalytic properties of the regenerated monomer and the similarities between the CD spectra of the monomer and RNase A. Wrongly paired RNase A is essentially devoid of enzymatic activity and exhibits an entirely different circular dichroism spectrum than native enzyme (Pflumm and Beychok, 1969; Schaffer, unpublished).

SCHEME I

$$P(SSG)_2 + 2GSH \rightleftarrows P(SH)_2 + 2GSSG \quad (1)$$

$$2P(SH)_2 + 2GSSG \rightleftarrows P(S\text{-}S)_2P + 2GSH \quad (2)$$

The regenerated bovine seminal monomer is extremely stable, and once it is produced the re-formation of native dimer is precluded. This suggests that under the conditions employed for regeneration the monomer is more stable than the dimer. The basis for preferential production of the monomer during regeneration becomes apparent upon examining the reactions involved in the conversion of the mixed disulfide monomer into the native dimer (Scheme I). In this scheme, $P(SSG)_2$. $P(SH)_2$, and $P(S\text{-}S)_2P$ represent the mixed disulfide monomer, the monomer with free sulfhydryls at cysteines-31 and -32, and the native dimer, respectively.

One striking feature of these reactions is the complex dependence upon the glutathione redox state. In reaction 1, GSH reduces the mixed disulfide species $P(SSG)_2$ to the free sulfhydryl form $P(SH)_2$. The formation of the native dimer from the free sulfhydryl would require GSSG, as can be seen in reaction 2. Thus, the redox potential of the two disulfide species ($P(SSG)_2$ and $P(S\text{-}S)_2P$) would control the direction of this reaction sequence.

Several lines of evidence indicate that the glutathione-mixed disulfide, $P(SSG)_2$, is the favored species in the presence of glutathione and that reaction 1 of Scheme I favors its formation. First, attempts to dimerize the glutathione-regenerated monomer in the presence of oxidized and reduced glutathione under oxidizing and reducing conditions all failed, although dimerization readily occurs from seminal RNase monomers obtained by selective reduction of the intermolecular disulfides with dithiothreitol(D'Alessio, unpublished). Second, the mixed disulfide species, $P(SSG)_2$, accumulates during glutathione-facilitated regeneration. Third, the intermolecular disulfide bonds of the native enzyme are selectively reduced at the same glutathione redox state used in the regenerations. This would suggest that $P(S\text{-}S)_2P$ is more readily reduced than $P(SSG)_2$. Thus, in the more reducing environment adequate to reduce $P(SSG)_2$ to $P(SH)_2$, $P(S\text{-}S)_2P$ would also be reduced. Under the less reducing conditions found optimal for the regeneration, $P(SSG)_2$ is favored.

Failure to regenerate native seminal RNase dimer with the glutathione redox system indicates the unique nature of this protein. Several monomeric and multimeric proteins have been regenerated with the glutathione redox system, and the major product of the regenerations has been nativelike protein (Wetlaufer and Ristow, 1973; Petersen and Dorrington, 1974; Ahmed et al., 1975).

IgG is the only protein with intermolecular disulfide bonds to be regenerated with the glutathione redox system. The intermolecular disulfides of IgG readily re-form from the dissociated monomers with either air oxidation or the glutathione redox system, while the intermolecular disulfide bonds of seminal RNase will only re-form with the air-regeneration system (Freedman and Sela, 1966; Parente et al., 1972; Petersen and Dorrington, 1974). This suggests a fundamental difference between the intermolecular forces of the two proteins and is supported by the following data. First, selective reduction of the intermolecular disulfide bonds with dithiothreitol causes partial dissociation of subunits in seminal RNase but not in IgG (Petersen and Dorrington, 1974; D'Alessio et al., 1975). Second, seminal RNase monomers alkylated at cysteine-31 and -32 do not associate (D'Alessio, unpublished data), while selectively reduced, alkylated, and dissociated IgG heavy and light chains do (Bigelow et al.,

1974). It follows from these data that the noncovalent intermolecular forces in seminal RNase are weaker than those in IgG. Thus, noncovalent association of seminal RNase is prevented by the glutathione-mixed disulfides at cysteine-31 and -32, while IgG subunit association and formation of intermolecular disulfides readily proceed in the presence of glutathione.

Glutathione is found in tissue at levels similar to those used in these studies (Tietze, 1969), and, although the concentration in semen has not been determined, it is known that seminal sulfhydryl content is high (D'Alessio, unpublished data). Since we have found glutathione to mediate selective reduction of the dimer and prevent dimerization of the monomer, we propose that in vivo dimerization must be potentiated by some other factor such as glutathione reductase (Harrap et al., 1973). The monomer–dimer interconversion takes on added significance, since the dimer appears to exhibit cooperative kinetics and substrate binding (D'Alessio, unpublished) and supported by the results here which show that the monomer is twice as active as the dimer. Moreover, the activity of the dimer toward double-stranded RNA and the RNA–DNA hybrid is greater than that of the monomer (Libonati et al., 1975). Thus, it might be expected that glutathione levels could regulate seminal RNase activity in vivo.

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