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Reversible Blocking of Half-Cystine Residues of Proteins and an Irreversible Specific Deamidation of Asparagine-67 of *S*-Sulforibonuclease under Mild Conditions†

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ABSTRACT: For use in protein-folding studies, a rapid procedure for the preparation of octa-*S*-sulforibonuclease A (SO_3-RNase A) with 2-nitro-5-(sulfothio)benzoate is described. The modification is specific for thiols and disulfide bonds. The modified protein was characterized and found to be enzymatically inactive and predominantly conformationally disordered. In the absence of thiols, the modified sulfhydryl groups were found to be stable over the pH range of 2–9. However, when the modified protein is incubated at neutral to slightly alkaline conditions for prolonged periods of time or at elevated temperatures, it undergoes a further (irreversible) modification that decreases its net charge at pH 8.0. Evidence is presented that demonstrates that this additional modification is due to the specific deamidation of asparagine-67. When incubated with an excess of reduced and oxidized glutathiones for 24 h at pH 8.2 and 25 °C, the reversible sulfo blocking group was removed, and essentially quantitative (94%) native enzymatic activity was regenerated from both SO_3-RNase A and its deamidated derivative (SO_3-RNase B). Although the two fully active refolded species differ in their elution behavior on ion-exchange chromatography, they are indistinguishable by many other methods. The significance of this finding for studies of the folding of RNase A is discussed.

There is much current activity using recombinant DNA techniques to produce various proteins. If the protein of interest contains disulfide bonds, it is necessary to pair the half-cystine residues properly to produce the native structure. While such a regeneration process is, in principle, a spontaneous one (Anfinsen, 1973), it is often hampered by practical difficulties, such as insolubility of the reduced material, and various methods have been proposed to fold the protein properly (Hayenga et al., 1983). However, such procedures are often cumbersome and, sometimes, are difficult to implement. It is, therefore, worthwhile to develop a suitable alternative procedure to obtain reversibly modified, soluble forms of the reduced protein that are chemically homogeneous,

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from which to begin refolding studies. This motivation led us to consider the use of reversible S-sulfonation to stabilize and solubilize a reduced protein. Therefore, as an illustration, we present here the preparation of SO_3-RNase A,[1] a study of its properties, and the regeneration of the native protein by treatment with a mixture of GSSG and GSH.

A second motivation of this work derives from the report that the experimental manipulations involved in the reduction of RNase A introduce one or more irreversible covalent modifications of the protein (Creighton, 1979). The cause(s) and site(s) of the chemical modification(s) have not yet been determined. Since such irreversible modification(s) could influence the interpretation of data from kinetic studies of the oxidative refolding pathway of RNase A (Konishi & Scheraga, 1980a,b; Konishi et al., 1981, 1982a–c; Scheraga et al., 1984), it is essential to determine the cause(s) and site(s) of the chemical modification(s). This purpose can also be achieved by studying the properties of SO_3-RNase A.

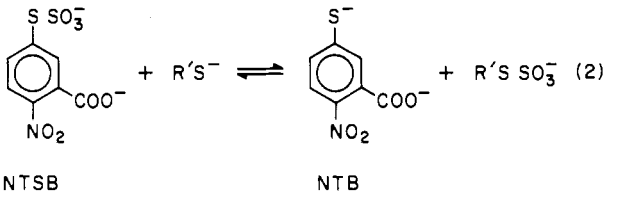
Sulfonation has been used previously to cleave disulfide bonds and to block the resulting thiol groups (Swan, 1957; Pechère et al., 1958; Bailey & Cole, 1959; Cole, 1967). The reaction proceeds under mild conditions and is specific for thiols and disulfide bonds. The ease with which the *S*-sulfo group can be removed is an additional advantage in that it enables the thiols to be blocked reversibly. For this reason, the *S*-sulfo blocking group was used in the synthesis of biologically active insulin (Katsoyannis et al., 1966) and RNase A (Gutte & Merrifield, 1969). Furthermore, Ruegg (1977) has reported that about 30% enzymatic activity can be regenerated from SO_3-RNase after first removing the SO_3 groups by reduction and then carrying out air oxidation. Although full recovery of enzymatic activity was not achieved in these experiments, they nevertheless suggest that SO_3-RNase might be a suitable starting material for investigations of the oxidative refolding pathway of this protein. Before adopting SO_3-RNase A as a suitable starting material for such studies, however, it is necessary to address several questions.

First, we require a convenient method for the preparation of SO_3-RNase A. Next, it must be demonstrated that the sulfonation reaction yields a chemically homogeneous product that is stable and conformationally disordered. Finally, it must be shown that *quantitative* recovery of native enzymatic activity is achieved after reoxidation and that the refolded, fully active enzyme is indistinguishable from the native form.

In the procedures currently used for the sulfonation of proteins, it is difficult to judge when the reaction is complete. In this paper, we report a modification of our recently reported analytical method for the quantitative determination of the concentration of disulfide bonds (Thannhauser et al., 1984) to prepare SO_3-RNase A. This procedure involves the cleavage of disulfide bonds of proteins with excess sulfite in the presence of a denaturant (Clarke, 1932; Cecil & McPhee, 1955; Stricks et al., 1955) as shown in eq 1. The thiol produced in this

$$RSSR' + SO_3^{2-} \rightleftharpoons RSSO_3^- + R'S^- \quad (1)$$

reaction is then reacted with disodium 2-nitro-5-(sulfothio)-benzoate (NTSB) according to eq 2. Since 1 mol of disulfide



NTSB NTB

produces 1 mol of NTB quantitatively, the number of moles of disulfide bonds reacted can be determined by measuring the amount of NTB produced (ϵ_{412} = 13 600 M^{-1} cm^{-1}; Ellman, 1959).

In this paper, we report a modification of this analytical procedure that yields preparative quantities of SO_3-RNase A. Since this preparative procedure also makes use of NTSB, the extent of sulfonation can be determined easily. The modified protein generated by this procedure is then characterized and found to be chemically homogeneous and predominantly conformationally disordered. We then describe a procedure to regenerate full enzymatic activity from SO_3-RNase A and demonstrate that the regenerated protein is indistinguishable from the native enzyme.

In addition, by investigating the pH dependence of the stability of SO_3-RNase A, we find that, with time or at elevated temperatures, this protein derivative is susceptible to the same irreversible covalent modification that occurs in reduced RNase A (Creighton, 1979). This covalent modification is shown to be a specific deamidation of asparagine-67. By determining the pH dependence of the rate of deamidation, we are able to estimate the degree of deamidation that may have arisen in kinetic studies of the refolding pathway of reduced RNase A (Konishi et al., 1981, 1982a,b) and judge whether this modification affected the results and conclusions reported in those investigations.

Experimental Procedures

Materials

Bovine pancreatic ribonuclease A, type IIA, obtained from Sigma Chemical Co., was purified by the method of Taborsky (1959). Tris base, barium cytidine cyclic 2′,3′-phosphate, and reduced and oxidized glutathione were also purchased from Sigma Chemical Co. Hydroxylamine hydrochloride was obtained from Mallinckrodt Chemical Co., and the Ellman reagent [5,5′-dithiobis(2-nitrobenzoic acid)], D_2O, [$^2H_4$]acetic acid, NaOD and DCl were obtained from Aldrich Chemical Co. Guanidine hydrochloride was obtained from Schwarz/Mann. NTSB was prepared by the method of Thannhauser et al. (1984). Carboxymethylcellulose (CM-52) was obtained from Whatman Ltd. and Bio-Gel P-6DG from Bio-Rad Laboratories. The isoelectric focusing gels were Ampholine Pagplates, pH range 3.5–9.5, obtained from LKB. The p*I* standard solution[2] and protein stain were obtained from Serva Chemical Co. The Mono-Q 5/5 (anion exchange), the Mono-S 5/5 (cation exchange), and the P-10 prepacked Sephadex G-25 desalting columns were purchased from Pharmacia Fine Chemicals.

The SO_3-RNase A was prepared as follows: 100 mg of chromatographically purified RNase A was dissolved in 5.0 mL of "cleavage solution" (0.3 M sodium sulfite and 6 M

[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; SO_3-RNase A, octa-*S*-sulforibonuclease; SO_3-RNase B, SO_3-RNase A that is deamidated at Asn-67; NTSB, disodium 2-nitro-5-(sulfothio)benzoate; NTB, 2-nitro-5-mercaptobenzoic acid; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; GSSG, oxidized glutathione; GSH, reduced glutathione; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Gdn·HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; C>P, barium cytidine cyclic 2′,3′-phosphate; p*I*, isoelectric point; NMR, nuclear magnetic resonance.

[2] The proteins (and the isoelectric points of their major bands, supplied by the manufacturer) that were used to estimate the p*I* of SO_3-RNases A and B are as follows: cytochrome *c*, 10.65; ribonuclease, 9.45; whale myoglobin, 8.3 and 7.7; horse myoglobin, 7.3 and 6.9; conalbumin, 5.9; β-lactoglobin, 5.34; bovine albumin, 4.7; ferritin, 4.4; amyloglucosidase, 3.5.

Gdn·HCl, adjusted to pH 8.0). The disulfide bond cleavage was allowed to proceed with stirring for 10 min. Then, 2.0 mL of "NTSB stock solution" (Thannhauser et al., 1984; 50 mM NTSB/1 M Na_2SO_3, pH 7.5) was added.

At 5-min intervals, 20-μL aliquots were removed and pipetted directly into cuvettes containing 3.0 mL of 0.1 M Tris-HCl, pH 8.0. The absorbance of this solution at 412 nm was measured on a modified Cary 14 spectrophotometer (Denton et al., 1982) to determine the extent of modification. The reaction was judged to be complete (with quantitative yields) after 10 min when there was no longer any increase in the absorbance at 412 nm (and for 10 min thereafter). The pH of the reaction was then adjusted to 3.0 with glacial acetic acid, and the volume was increased to 14 mL by the addition of deionized water. The protein was then desalted on a 2.5 × 35 cm Bio-Gel P-6DG column, with 0.1 N acetic acid as the eluant. The fractions containing protein were pooled and lyophilized. The lyophilized protein was stored in a desiccator at −20 °C.

Methods

Chromatography. The purity of the SO_3-RNase was determined by high-performance anion-exchange chromatography on a Spectra Physics Model 8700 or 8000 liquid chromatograph equipped with a prepacked Mono-Q 5/5 column (Pharmacia). The column was equilibrated at room temperature with 20 mM Tris-HCl adjusted to pH 8.0, and the protein was eluted at 1.0 mL/min with a linear gradient (0–0.3 M NaCl) in 20 min.

This chromatographic procedure was also useful as a check of the stability of the modified protein. The protein (1 mg/mL) was incubated for 24 h at 38 °C at various pHs from 2 to 9. The buffer was 25 mM sodium phosphate and 25 mM sodium citrate, adjusted to the desired pH with sodium hydroxide. After incubation, the protein was rapidly desalted with a P-10 desalting column that had previously been equilibrated with 20 mM Tris-HCl, pH 8.0. The fraction containing the protein was collected and stored at 0 °C until it could be analyzed by the chromatographic method described above. The two major protein-containing fractions (SO_3-RNase A and SO_3-RNase B of Figure 1) revealed by this chromatographic analysis were collected and saved for later analysis.

Molecular Weight. The molecular weight of SO_3-RNase (A and B) was determined by the sedimentation equilibrium method at 27 °C (Schachman, 1959) with a Beckman Model E analytical ultracentrifuge equipped with UV optics. The protein (3.0 mg/mL) was dissolved in 0.1 M sodium phosphate, pH 8.0, and dialyzed for 72 h at room temperature against 1 L of 0.1 M sodium phosphate, pH 8.0. The dialyzate was used as the reference solution in the ultracentrifuge cell. To check for small amounts of aggregation not easily detectable by the sedimentation equilibrium method, the sedimentation velocity method was employed (Schachman, 1959). For these experiments a Beckman Model E ultracentrifuge equipped with interference optics was used. The sample was prepared by dissolving 9.9 mg of SO_3-RNase in 1.0 mL of 0.1 N Tris-HCl, adjusted to pH 8.0.

Completion of Reaction. The presence of any incomplete reaction products (any free thiols or disulfide groups) was checked by the method of Thannhauser et al. (1984).

Isoelectric Point. An estimate of the isoelectric points of SO_3-RNase A and SO_3-RNase B was obtained by analytical isoelectric focusing in the pH range of 3–9.5 on a Bio-Rad Model 1415 isoelectric focusing cell equipped with an EC500 power supply. A sample of myoglobin (at a higher concentration than in the p*I* standard solution)[2] was included in each run to help determine the exact point of focusing. To facilitate the estimation of the p*I*, a p*I* standard solution (a solution of nine proteins of known p*I*'s)[2] was included in each gel. The gels were stained with Serva blue stain according to the procedures recommended by the manufacturer.

Check for Deamidation at Asn-67. SO_3-RNase A and SO_3-RNase B were treated with hydroxylamine according to the procedure of Bornstein & Balian (1970). This reaction is known to cleave RNase A specifically at the Asn-67–Gly-68 peptide bond (Bornstein & Balian, 1970). The hydroxylamine-treated protein was then fractionated on a Sephadex G-50 superfine column (1.5 × 93 cm), equilibrated with 70 mM Tris-HCl at pH 8.0. The protein was eluted at a flow rate of 9.0 mL/h.

Nuclear Magnetic Resonance Spectroscopy. The ^1H NMR spectra were recorded at 300 MHz on a Brüker WM-300 spectrometer. Samples of native RNase A at 10 mg/mL were prepared in D_2O. The apparent pH was adjusted to 3.0 with minute amounts of DCl and NaOD. Samples of SO_3-RNase A of 10 mg/mL were prepared in 0.1 M [$^2H_4$]acetic acid and adjusted to an apparent pH of 3.0 with NaOD. The NMR spectra were recorded at 22 °C and referenced to external DSS contained in a capillary insert. The spectra were resolution-enhanced by transformation to a Gaussian free-induction decay prior to Fourier transformation (Ferrige & Lindon, 1978).

Regeneration. The regeneration of native enzymatic activity from the SO_3-RNase A was accomplished as follows: 0.1 mL of a solution 10 mM in GSSG, 20 mM in GSH, and 0.1 N in Tris-HCl, pH 8.2, was added to each of four 1.0-mL samples containing 0.5 mg/mL SO_3-RNase A in 0.1 N Tris-HCl and 3 mM EDTA, pH 8.2. The final GSSG and GSH concentrations were 1 and 2 mM, respectively. To a fifth 1.0-mL sample of 0.5 mg of SO_3-RNase A, 0.1 mL of 0.1 N Tris-HCl, pH 8.2, was added. A sample containing 0.1 mg of SO_3-RNase B in 1.0 mL was regenerated by an identical procedure. The six solutions were allowed to stand at room temperature for 24 h. After this time, 0.01-mL aliquots were removed from each sample and pipetted directly into 3.0 mL of 0.45 mM C>P (0.2 mg/mL) in 20 mM sodium acetate, pH 5.5. The enzymatic activity of the SO_3-RNase A (or B) and of regenerated RNase A toward C>P was monitored by the increase in optical density at 286 nm (Crook et al., 1960). The percent recovery of activity was computed as the ratio of the specific activity of the regenerated material to that of the original unmodified enzyme.

The regenerated RNase A was separated from the GSSG and the GSH by gel filtration chromatography on a Bio-Gel P-6DG column (1.5 × 30 cm) equilibrated with 0.1 N acetic acid. The fractions corresponding to RNase A were collected, lyophilized, and stored at 4 °C for subsequent analyses.

The purity of the regenerated protein was checked by high-performance cation-exchange chromatography on a Mono-S 5/5 column (Pharmacia) equilibrated with 25 mM sodium phosphate, pH 6.5. The column was eluted at 1.0 mL/min with a linear gradient of 0–0.3 M sodium chloride in 20 min.

The ultraviolet absorption spectra of SO_3-RNases A and B and of regenerated RNase A in water were recorded on a modified Cary 14 spectrophotometer (Denton et al., 1982).

The regenerated RNase A was checked for the presence of any covalently linked aggregates by its electrophoretic mobility on a linear 5–15% polyacrylamide–SDS slab gel under non-reducing conditions (Laemmli, 1970). Its mobility was compared to that of the native enzyme run on the same gel. The

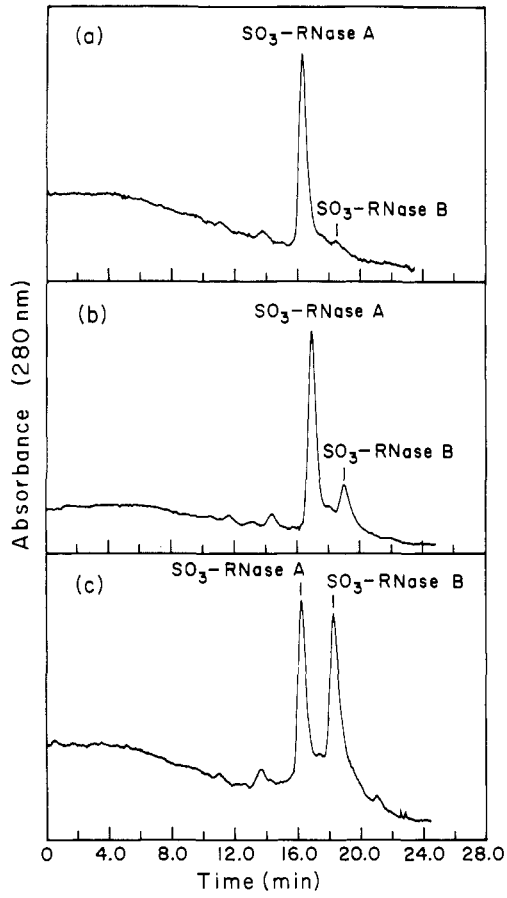


FIGURE 1: Elution profiles of several preparations of SO_3-RNase A on a Mono-Q 5/5 (anion-exchange) column in 20 mM Tris-HCl, pH 8.0. The samples had been preincubated for 24 h at 38 °C and pH (a) 6.0, (b) 7.5, and (c) 8.5. The chromatograms were developed at 1.0 mL/min with a linear gradient of sodium chloride (0.0–0.3 M) in 20 min.

gel was stained with Coomassie brilliant blue G-250 (Fairbanks et al., 1971).

Amino Acid Analysis. Amino acid analysis was performed on a Technicon TSM amino acid autoanalyzer. The samples were hydrolyzed according to the method of Swadesh et al. (1984b).

Concentration. All protein concentrations were determined by micro Kjeldahl nitrogen analysis (Noel & Hambleton, 1976). The concentration of native RNase A was checked routinely spectrophotometrically with $\epsilon_{277.5} = 9800\ M^{-1}\ cm^{-1}$ (Sela & Anfinsen, 1957).

Results

SO_3-RNase A that had been freshly prepared by the procedure recommended here eluted as a single symmetric peak when analyzed by high-performance anion-exchange chromatography (Mono-Q). This indicates that the sulfonation reaction proceeds quantitatively to give a single pure product. However, after incubation of the originally homogeneous SO_3-RNase A at 38 °C at neutral to alkaline pH for 24 h, an additional more negatively charged species (SO_3-RNase B) appears (Figure 1). The reaction that produces SO_3-RNase B was found to be irreversible. It appears to be first order in protein concentration because its half-time is independent of the initial protein concentration. It should be noted that no significant earlier (more positively charged) species were generated on incubation. The rate at which SO_3-RNase A is converted to SO_3-RNase B was found to depend on the pH of incubation. A plot of the common logarithm of the observed first-order rate constant of deamidation vs. pH revealed a straight line of slope 0.3 with an intercept of −3.93, with a linear correlation coefficient of 0.998.

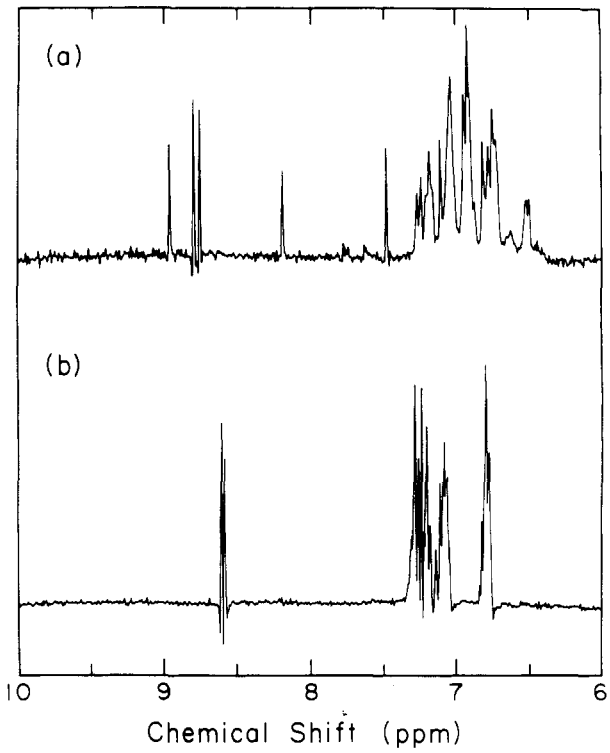


FIGURE 2: ^1H NMR spectra at 300 MHz (at 22 °C) of (a) native RNase in D_2O at an apparent pH of 3.0 and (b) SO_3-RNase A in 0.1 N [$^2H_4$]acetic acid in D_2O at an apparent pH of 3.0. The chemical shifts are referenced with respect to external DSS.

The molecular weight of SO_3-RNase (A and B) was 13 000 or 14 000, depending on the value used for the partial specific volume, viz., 0.695 or 0.703 mL/g, respectively (Richards & Wyckoff, 1971). This is in good agreement with the formula weight of 14 331. The sedimentation velocity run revealed no detectable aggregates at a protein concentration of 9.9 mg/mL at pH 8.0. The protein sedimented as a single symmetrical band, with $s = 1.63$ S at 27 °C.

Figure 2 shows the aromatic region (δ = 6.5–9.0 ppm) of the ^1H NMR spectra at 300 MHz of both native and SO_3-RNase A (10 mg/mL) at 22 °C and pH 3.0. It is obvious that the S-sulfonation of RNase A has caused large changes in the chemical shifts of most of the resonances shown. The ^1H NMR spectrum of SO_3-RNase A is similar to those of both heat-denatured (Benz & Roberts, 1975) and disulfide-reduced RNase A (Konishi & Scheraga, 1980b). From these ^1H NMR data (Figure 2), we conclude that, like heat-denatured or disulfide-reduced RNase, SO_3-RNase A is predominantly disordered. ^1H NMR data presented elsewhere (Swadesh et al., 1984a) have demonstrated the presence of a local short-lived structure in SO_3-RNase A. No thiols or disulfides were detectable with NTSB (Thannhauser et al., 1984) in samples of freshly prepared SO_3-RNase A or samples that contained as much as 50% SO_3-RNase B.

Freshly prepared solutions of SO_3-RNase A gave a single band when analyzed by isoelectric focusing in the pH range of 3.0–9.5. The p*I* of this band was estimated from a least-squares fit of the distance from the cathode to that of the proteins of known p*I* in the standard solution run in the same gel. It was found to be 5.7 ± 0.2, which agrees with the predicted value of 5.7. When the sample solution used for isoelectric focusing was found to contain measurable amounts

Table I: Enzymatic Activity of Various Preparations

sample	sp act. (units/mg)	recovery (%)
R-1[a]	1676	87
R-2[a]	1809	94
R-3[a]	1809	94
R-4[a]	1900	99
R-B[a,b]	1809	94
SO_3-RNase	0	
native RNase A	1923	

[a] Each of the samples was regenerated (independently) from the *S*-sulfo protein. [b] This sample contained only SO_3-RNase B that had been isolated by high-performance anion-exchange chromatography (Figure 1) from a solution of SO_3-RNase A after incubation at 38 °C, pH 8.0, for 24 h.

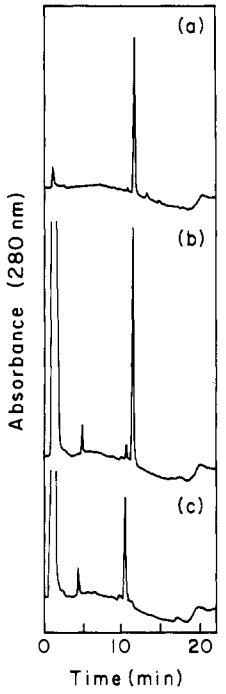


FIGURE 3: Comparison of the elution profiles of (a) native RNase A, (b) a regenerated sample of SO_3-RNase A, and (c) a regenerated sample of SO_3-RNase B, obtained by cation-exchange chromatography on a Mono-S 5/5 column. The column was equilibrated at pH 6.5 with 25 mM sodium phosphate, and the protein was eluted at 1.0 mL/min with a linear gradient of 0–0.3 M NaCl in 20 min.

of SO_3-RNase B on anion-exchange chromatography, a second band of lower p*I* was found. The p*I* of this second band was 5.35 ± 0.2, which is consistent with a predicted value of 5.4 for a monodeamidated SO_3-RNase A.

Table I shows the measured enzymatic activity toward C>P of SO_3-RNase, four regenerated samples of SO_3-RNase A, a regenerated sample of SO_3-RNase B, and the native enzyme. The SO_3-RNase A was found to be devoid of enzymatic activity, while the specific activity of the four regenerated samples of SO_3-RNase A averaged 94% of that of the native enzyme. The more negatively charged species, SO_3-RNase B, was likewise found to generate 94% of its native activity.

Figure 3 shows the chromatographic behavior of (a) native RNase A, (b) RNase A regenerated from SO_3-RNase A, and (c) the fully active protein that was regenerated from SO_3-RNase B. The peaks at 1 and 4 min in Figure 3b,c are due to oxidized/reduced glutathione and Tris buffer, respectively, which were not removed before carrying out this analysis. It can be seen that the mobilities of the major species in Figure 3a,b are identical. The earlier eluting peak at 11 min in Figure 3b is presumably due to a small amount of SO_3-RNase B derived from SO-RNase A during the refolding process, since

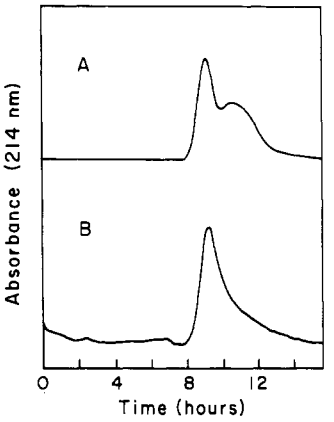


FIGURE 4: Elution pattern of peptides in gel filtration on a 1.5 × 93 cm Sephadex G-50 column after hydroxylamine treatment of (A) SO_3-RNase A and (B) SO_3-RNase B. The column was equilibrated at pH 8.0 with 70 mM Tris-HCl and eluted at a flow rate of 9 mL/h.

Table II: Amino Acid Analysis of Several Preparations

	SO_3-RNase A	SO_3-RNase B[a]	regenerated RNase[b]
Asx	15.2	15.0	15.0 (15)
Thr	9.7	9.4	9.4 (10)
Ser	13.6	12.8	13.1 (15)
Glx[c]	12.3	12.1	11.9 (12)
Pro	3.8	4.4	4.0 (4)
Gly	3.1	3.6	3.0 (3)
Ala[c]	11.7	11.9	12.1 (12)
Cys	7.2	7.1	7.9 (8)
Val	8.9	9.0	8.8 (9)
Met	3.9	4.1	4.1 (4)
Ile	2.3	2.1	2.3 (3)
Leu	2.0	2.1	2.2 (2)
Tyr	5.8	5.5	5.7 (6)
Phe	2.7	3.0	3.0 (3)
His	4.2	4.3	4.1 (4)
Lys	10.4	10.5	10.2 (10)
Arg	3.9	3.8	3.9 (4)

[a] This material was isolated by high-performance anion-exchange chromatography (Figure 1) from a solution that had been incubated at 38 °C and pH 8.0 for 24 h. [b] Theoretical values are given in parentheses. [c] Glx and Ala were taken as 24 in computing the values in this table.

it elutes in the same position as the major 11-min peak in Figure 3c.

SO_3-RNase A exhibited a UV absorption maximum at 275 nm with a molar extinction coefficient of 8500 M^{-1} cm^{-1}. The change in the absorbance maximum from 277.5 nm in native RNase A to 275 nm is characteristic of several denatured forms of RNase (Anfinsen, 1956; Haber & Anfinsen, 1962). The UV spectrum of the regenerated RNase A showed the characteristic absorbance maximum at 277.5 nm and is identical with that of native RNase A (Anfinsen, 1956; Haber & Anfinsen, 1962).

The results of the SDS–polyacrylamide gel electrophoresis showed that the regenerated material is homogeneous in molecular weight and has the same electrophoretic mobility as a sample of the native molecule run on the same gel. There were no detectable covalent aggregates.

Figure 4A shows the elution pattern of the peptides generated by the hydroxylamine treatment of pure SO_3-RNase A. The pattern observed is similar to that reported by Bornstein & Balian (1970) for carboxymethylated RNase. Figure 4B shows the elution pattern generated by hydroxylamine treatment of SO_3-RNase B. As can be seen, the amount of cleavage is drastically reduced. The elution pattern in this

case is similar to that obtained from (carboxymethyl)RNase A that had been treated with base (Bornstein & Balian 1970).

Table II gives the results of the amino acid analysis of SO_3-RNase A, SO_3-RNase B, and regenerated RNase A. As can been seen from the table, there is apparently no acid-stable modification of the protein.

Discussion

Properties of SO_3-RNase A. As demonstrated by the results of high-performance anion-exchange chromatography and the single band observed on analytical isoelectric focusing, the sulfonation reaction proceeds to a single, chemically homogeneous derivative. It should be pointed out that, although this paper deals only with RNase A, a similar analytical procedure was found to be quantitative for a wide variety of proteins (Thannhauser et al., 1984). This suggests that this preparative sulfonation procedure should be applicable generally and may prove useful for industrial biotechnological applications that currently employ the older and relatively cumbersome sulfonation procedures (Hayenga et al., 1984).

Figure 1 indicates that, on incubation at 38 °C and neutral to alkaline pH for 24 h, SO_3-RNase A is partially converted to a second more negatively charged species designated as SO_3-RNase B. The origin of SO_3-RNase B will be discussed below. It should be noted here, however, that it is *not* due to sulfonation of residues other than cysteine and cystine because the modification occurs after all the sulfonation reagents have been removed. The absence of any significant earlier eluting (more positively charged) species before SO_3-RNase A in Figure 1 demonstrates the stability of the SO_3^- blocking group in the pH range studied. The loss of one or more SO_3^- groups to yield a thiol or disulfide bond would increase the charge on the protein and result in the accumulation of earlier eluting species. The very minor amounts of earlier eluting species observed in Figure 1 may be due to the loss of blocking groups; however, when these solutions were checked with NTSB (Thannhauser et al., 1984), they were found to contain *no* detectable thiols or disulfide bonds . These findings are contrary to an earlier report by Cole (1967), who found the *S*-sulfo blocking group to be stable only at neutral pH, but are in accord with a more recent report by Ruegg (1977).

Characterization of SO_3-RNase A indicates that it is predominantly conformationally disordered. This is demonstrated by its complete loss of enzymatic activity and by the ultraviolet absorption and NMR spectra, both of which show the changes characteristic of heat-denatured or disulfide-reduced RNase A (Anfinsen, 1956; Haber & Anfinsen, 1962; Benz & Roberts, 1975; Konishi & Scheraga, 1980b). The fact that the measured p*I* (5.70) of this derivative is the same as that computed from the p*K*'s of its ionizable groups (with no allowance for environmentally induced alterations of p*K*'s) suggests that the p*K*'s of the ionizable groups have been normalized; i.e., these groups are exposed to the solvent. Furthermore, a recent report (Thannhauser et al., 1985) demonstrates that SO_3-RNase A is completely susceptible to digestion by several proteases. These results clearly indicate that the sulfonation procedure has caused a dramatic conformational change in the protein and results in a predominantly disordered ensemble of conformations. This finding is in agreement with the known properties of both performic acid oxidized and reduced and carboxymethylated RNases (Harrington & Sela, 1959).

To initiate the refolding reaction from SO_3-RNase A, it is first necessary to remove the sulfonates. This may be accomplished by their nucleophilic displacement with a sufficient excess of a thiol such as GSH. This treatment produces the fully reduced protein, which can then be oxidized to the native enzyme with air (Ruegg, 1977) or presumably by the addition of an oxidant such as GSSG. Alternatively, as was done here, the native enzyme can be regenerated *directly* from SO_3-RNase A. This was accomplished by the simultaneous addition of both GSH and GSSG to a solution of SO_3-RNase A. This procedure generates reduced species that oxidize and refold to the native enzyme in a concerted process. Full enzymatic activity was regenerated from SO_3-RNase A when treated in this manner. This shows that regeneration is accompanied by a reversal of the conformational change, leading to a refolded molecule that has the same active site geometry as the native enzyme. Insofar as the active site geometry is determined by the conformation of the rest of the molecule, it can reasonably be concluded that the regenerated molecule is structurally very similar to the native molecule. This conclusion is supported by the fact that 95% of the regenerated protein has the same chromatographic mobility as the native molecule (Figure 3), demonstrating that it has the identical surface charge, and it shows the same characteristic ultraviolet absorption spectrum, demonstrating that the average environments of the tyrosyl residues are the same in both species.

Approximately 5% of the protein regenerated from the originally homogeneous SO_3-RNase A was found to elute earlier than the native protein when analyzed by high-performance cation-exchange chromatography (Figure 3b). This indicates that it is more negatively charged and suggests that the process responsible for the generation of SO_3-RNase B in Figure 1 can also occur during the regeneration reaction or in the native protein. This hypothesis is supported by the finding that the protein regenerated from chromatographically purified SO_3-RNase B eluted at the same position as this fraction (Figure 3c). In a separate experiment (not shown here), the protein regenerated from purified SO_3-RNase B and the "contaminant" in the material regenerated from SO_3-RNase A coeluted when the two were chromatographed together.

Origin of SO_3-RNase B. The fact that this modification decreases the net charge on the protein and occurs at neutral to alkaline pH immediately suggests deamidation as its origin. The measured p*I* of SO_3-RNase B, 5.35, tends to support the deamidation hypothesis because it is consistent with a predicted value of 5.4 for monodeamidated SO_3-RNase A. Base-catalyzed deamidation is known to occur under the conditions used here but generally at much slower rates (Robinson, 1974). Since the rate of deamidation is sequence-dependent (Robinson 1974), the existence of a particularly labile sequence can possibly explain the discrepancy in rates. Concerning this point, it is relevant to note that Bornstein & Balian (1970) have demonstrated that the single Asn-Gly sequence (residues 67 and 68) in RNase A is uniquely susceptible to peptide chain cleavage by hydroxylamine due to its ability to form an intrachain cyclic imide as shown in Figure 5a. Chain cleavage occurs by a nucleophilic attack of hydroxylamine at *either* of the carbonyl carbons of the imide, followed by an internal rearrangement to a bicyclic intermediate that decomposes to liberate a new free amino group and a mixture of α- and β-hydroxamates (Bornstein & Balian, 1977). In the absence of hydroxylamine, the cyclic imide is readily hydrolyzed to both α- and β-linked aspartic acids as shown in Figure 5b. Once this hydrolysis has occurred, the possibility of chain cleavage with hydroxylamine is greatly reduced because α-*Asp*-Gly and β-*Asp*-Gly sequences do not readily form the cyclic imide (Bornstein & Balian, 1977). In light of this evidence, Asn-67 can be expected to be particularly labile to base-catalyzed deamidation. It is presumably this process that is responsible

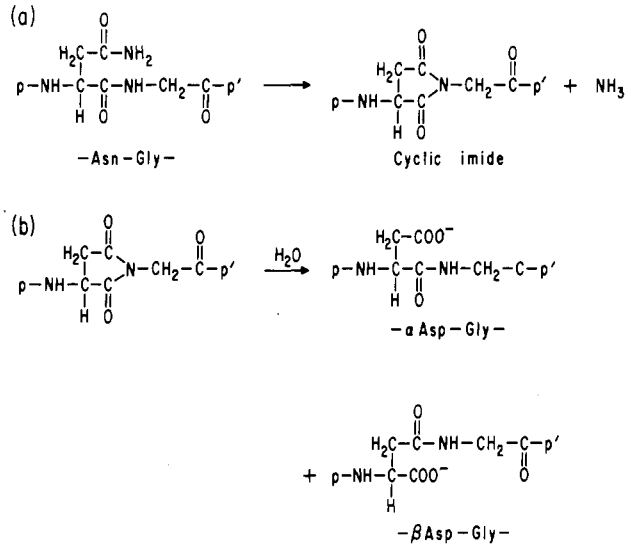


FIGURE 5: (a) Formation of cyclic imide in Asn-Gly sequence. (b) Formation of α- and β-Asp-Gly linkages from the hydrolysis of the cyclic imide.

for the production of SO_3-RNase B. If this is so, then SO_3-RNase B should be resistant to cleavage by hydroxylamine. This expectation is confirmed by the chromatograms of Figure 4, where Figure 4A demonstrates that SO_3-RNase A is readily susceptible to cleavage by hydroxylamine while Figure 4B shows that SO_3-RNase B is highly resistant to this nucleophile. It can therefore be concluded that SO_3-RNase B has undergone at least a deamidation at asparagine-67.

The plot of the common logarithm of the first-order rate constant of deamidation vs. pH did not have a slope of 1, indicating that the reaction is not first-order in OH concentration. The undoubtedly because the reaction mechanism is complex. This is undoubtedly because the reaction mechanism is complex. This is consistent with the deamidation mechanism proposed by Bornstein & Balian (1977) involving an intermediate and more than one product (i.e., α- and β-Asp).

It is possible that SO_3-RNase B is also deamidated or otherwise modified at residues in addition to Asn-67, but this is considered unlikely for the following reasons. The modification reaction proceeds to a single product as indicated by Figure 1. If there were more than a single modification involved, then one would expect to observe the accumulation of several products, each more negatively charged than SO_3-RNase A, corresponding to the mono-, di-, and triderivatized species. This is obviously not the case. The fact that essentially quantitative (Table I) recovery of enzymatic activity can be regenerated from preparations of SO_3-RNase B is seen as further evidence that it is modified at only a single amide, on the basis of the recent work of Venkatesh & Vithayathil (1984). They reported that several *mono*deamidated derivatives of RNase A isolated from *highly acidic* solutions were found to be fully active. Derivatives with more than a single deamidation had drastic reductions in their enzymatic activities. It is also relevant to note that the monodeamidated derivatives were found to refold to fully active species after reduction, although at slower rates than reduced native RNase A (Venkatesh & Vithayathil, 1985). It is important to note that Venkatesh & Vithayathil (1985) used highly acidic conditions to achieve deamidation; we wish to emphasize here that Asn-67 in RNase A can be deamidated even under mild conditions (e.g., pH 6.5).

The properties of SO_3-RNase B are similar to those of the more negatively charged species of reduced RNase A reported by Creighton (1979). In addition, both of these species appear at the same rate under similar conditions. These observations suggest that they have the same origin, the deamidation of Asn-67. The susceptibility of this residue to deamidation depends on the amino acid sequence of the protein. Therefore, deamidation of Asn-67 can be expected to occur at a similar rate in all unfolded forms of this protein.

Implications for Folding Studies. The susceptibility of Asn-67 to deamidation could influence the kinetics of the oxidative refolding of RNase A, as in the experiments of Konishi & Scheraga (1980a,b) and Konishi et al. (1982a,b). The multiphasic refolding kinetics observed in these studies could be explained either by chemical heterogeneity (Creighton 1984) or by multiple refolding pathways characterized by different rate-limiting steps (Konishi et al., 1982a–c; Scheraga et al., 1984). In order to distinguish between these possibilities, it is necessary to know the percentage of deamidated species in the protein samples studies. By using the data on the pH dependence of the first-order rate constant and unpublished data on the dependence of the rate of deamidation on temperature, we estimate that only 3–5% of the protein could have been deamidated under the conditions used by Konishi & Scheraga (1982a,b). Such a small amount of deamidation is not likely to have affected the results and conclusions of these studies. Only a relatively minor amount of deamidation arose in these studies because very mild conditions were used for reduction and refolding, viz., 25 °C, pH 7.8–8.0. Larger amounts of deamidation can be expected if more severe conditions are used (Creighton, 1979).

RNase A that has been specifically deamidated at Asn-67 is useful in another connection since this modification may be regarded as a point mutation. It is an especially attractive mutation for study because it occurs in a region of the protein sequence that has been suggested as a possible chain-folding initiation site (Burgess & Scheraga, 1975; Anfinsen & Scheraga, 1975; Chavez & Scheraga, 1980a,b; Montelione et al., 1984) and it occurs in the vicinity of cysteine residues 65 and 72, which are involved in the first preferred disulfide pairing (Takahashi & Ooi, 1976). By comparing the refolding behavior of these variants (one with an α-linked Asp in position 67, the other with a β-linked Asp) to that of the native protein, it should be possible to determine the importance of this region of the sequence in the refolding pathway.

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Registry No. NTSB, 90577-18-7; GSSG, 27025-41-8; GSH, 70-18-8; Asn, 70-47-3; RNase, 9001-99-4.

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