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Biochemistry

Expression of wild-type and mutant bovine pancreatic ribonuclease A in *Escherichia coli*

(fusion protein/inclusion bodies/factor Xa/disulfide mutants)

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ABSTRACT **Wild-type ribonuclease A and five mutants thereof have been expressed in *Escherichia coli* as fusion proteins by using a T7 expression system. The five mutants are C[65-72]S, C[40-95]S, C[58-110]S, C[26-84]S, and K41G. The expressed fusion protein formed inclusion bodies which were then cleaved by factor Xa. The cleaved ribonuclease A was isolated as unfolded (sulfonated), soluble protein which was subsequently folded. This expression system can be used to produce mutants of ribonuclease A in yields suitable for folding and structural studies. All four native three-disulfide mutants exhibited enzymatic activity (5–30%), although only two were thermally stable at room temperature, demonstrating that no single native disulfide bond is essential for folding. The K41G mutant was enzymatically inactive with cyclic cytidine monophosphate as substrate.**

Bovine pancreatic ribonuclease A (RNase A) has been used as a model system to investigate the pathways of protein folding and the role played by the disulfide bonds in the regeneration of the native protein from the reduced form (1–13). Such studies could be facilitated by recombinant DNA methods to introduce ^{15}N or ^{13}C labels for multidimensional NMR spectroscopic studies of trapped intermediates along the folding pathways. In addition, by replacing each of the native disulfide-forming cysteine pairs, in turn, it is possible to obtain material to investigate the influence of each disulfide bond on the thermal stability of the protein. This methodology can also be used to introduce mutations in other regions of the polypeptide chain to study features of the folding process—e.g., the role of cis–trans isomerization about peptide bonds preceding proline (14) or the interactions that other residues contribute in forming chain-folding initiation sites (15) and in stabilizing folding intermediates.

Breslow *et al.* (16) have developed cyclodextrin mimics of ribonuclease involving only histidines, and no lysine, in the catalysis of RNA. However, Lys^{41} has previously been thought to be involved in catalysis by increasing the electrophilicity of the phosphorous and stabilizing a trigonal bipyramidal intermediate (ref. 17, p. 432). In addition, selective blocking of Lys^{41} by chemical methods, such as amidination (ref. 17, p. 326) or crosslinking to Lys^{7} (18), has been shown to inactivate the enzyme. Therefore, it is of interest to mutate Lys^{41} and examine the catalytic properties of this enzyme.

For these reasons, we have developed an efficient expression system to produce mutants of RNase A with which to investigate the structural and functional features of this enzyme.

Several expression systems for RNase A have been developed previously (14, 19–22). Some of these systems direct the export of the target protein to the periplasmic space or even to the extracellular medium (unpublished observations; refs. 19 and 20). These secretion systems produce authentic recombinant RNase A with the correct N-terminal sequence but require that the expressed protein be folded *in vivo*. Therefore, mutations of amino acids which are important in stabilizing the folding protein result in low expression yields. In addition, expression systems that produce folded, active RNase A, even when directed to the extracellular medium or periplasmic space, have been observed to give extremely low yields, presumably because of the inherent toxicity of active RNase A (unpublished results). Other systems express the target protein in the cytosol, modified with a methionine at the N terminus, but again, such active RNase A is likely to be produced in very low yields. The formation of inclusion bodies protects the expressed RNase A from degradation by intracellular proteolysis. However, inclusion bodies have to be solubilized in either folded or unfolded form in order to be purified.

Here, cysteines were blocked reversibly with a sulfonating reagent that can prevent formation of intermolecular disulfide bonds or aggregation of unfolded wild-type or mutant RNase A. In this paper, we present a procedure to obtain pure, unfolded recombinant RNase A with the correct N-terminal sequence. This material is obtained from a factor Xa-cleavable fusion protein with a modified T7 gene 10 protein that is expressed in intracellular inclusion bodies. This system provides high-level expression of mutant RNase A in an unaggregated and unfolded form. Since the ability to refold wild-type sulfonated RNase A (SO_3-RNase A) quantitatively has been well documented (13, 23), the protein obtained from this system is ideal for folding studies involving desired mutants of RNase A. We discuss here the expression of wild-type and mutant RNase A in terms of yield, activity, and some physical characterization of those mutants that formed stable folded structures at room temperature. The species described are the four native three-disulfide mutants C[65-72]S, C[40-95]S, C[58-110]S, and C[26-84]S, with cysteines replaced by serines, and K41G, which contains a glycine-for-lysine mutation near the active site of RNase A.

MATERIALS AND METHODS

Materials. A synthetic RNase A gene (cloned into a pGX22314 vector) was kindly provided by Genex (24). All enzymes were purchased from United States Biochemical or Promega. Mutagenesis was carried out with the T7-Gen *in vitro* mutagenesis kit (United States Biochemical). Oligonucleotides were synthesized by the Cornell Biotechnology Center (Ithaca, NY). pGEMEX-2 and phagemid R408 were from Promega. *Escherichia coli* strain HMS174(DE3)-pLys(S) was from Novagen (Madison, WI). *E. coli* strains JM109, JM101, and SURE were used as host cells for cloning and mutagenesis. Factor Xa was from Boehringer Mann-

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Abbreviations: cCMP, cyclic cytidine monophosphate; SO_3-RNase A, sulfonated RNase A.

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heim. Control nonrecombinant RNase A from Sigma was purified further on a column packed with S-Sepharose (Pharmacia) resin. HPLC columns were obtained from Pharmacia LKB.

Construction of Expression Vector. A plasmid was constructed to express RNase A as a fusion protein with a gene 10 protein by using pGEMEX-2, which utilizes a T7 expression system (25). A synthetic RNase A gene was originally cloned into the multiple cloning site of an M13 replicative-form bacteriophage vector from pGX22314 (a vector obtained from Genex). A factor Xa site was then introduced directly in front of the synthetic RNase A gene by oligonucleotide-directed mutagenesis, to create M13sj142. This gene was then subcloned from M13sj142 into the multiple cloning site of a modified pGEMEX-2 vector at the *Eco*RI/*Hin*dIII site, in frame and directly behind the gene coding for the T7 gene 10-II protein (Fig. 1). Two minor factor Xa recognition sites in gene 10 with the sequence Gly-Arg-Xaa (Arg^{68} and Arg^{279}) were changed in pGEMEX-2 to Gly-Asp-Xaa by oligonucleotide-directed mutagenesis using phagemid-prepared single-stranded DNA as a template (the modified pGEMEX-2 is now called pSJIIGEM-2, and gene 10 is now called gene 10-II) (Fig. 1). All subsequent mutants were constructed from the M13sj142 vector and subcloned into pSJIIGEM-2. All clones were sequenced by the dideoxy sequencing method with a kit from United States Biochemical.

Protein Expression. *E. coli* strain HMS174(DE3)-pLys(S) was used to express both wild-type and mutant fusion protein. This strain contains a plasmid, pLys(S), which directs the expression of T7 lysozyme, an inhibitor for T7 RNA polymerase (25), to control the basal expression level of T7 RNA polymerase. Ten milliliters of LB medium containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) was inoculated with a single colony at 37°C and agitated with a gyratory shaker overnight at 225 rpm. This culture was then diluted to 1 liter and allowed to incubate under the same conditions. At OD_{600} = 0.4, expression of protein was induced by gradual addition of 0.5 mM isopropyl β-D-thiogalactopyranoside over 2.5 hr. After 5–7 hr of further incubation (Fig. 2, lane 1), the cells were harvested by centrifugation at 6000 rpm with a Beckman JA-10 rotor for 30 min. The cell pellets were then resuspended in 50 ml of lysis buffer (25 mM Tris·HCl/5 mM EDTA/2 mM phenylmethylsulfonyl fluoride, pH 8.0) (26).

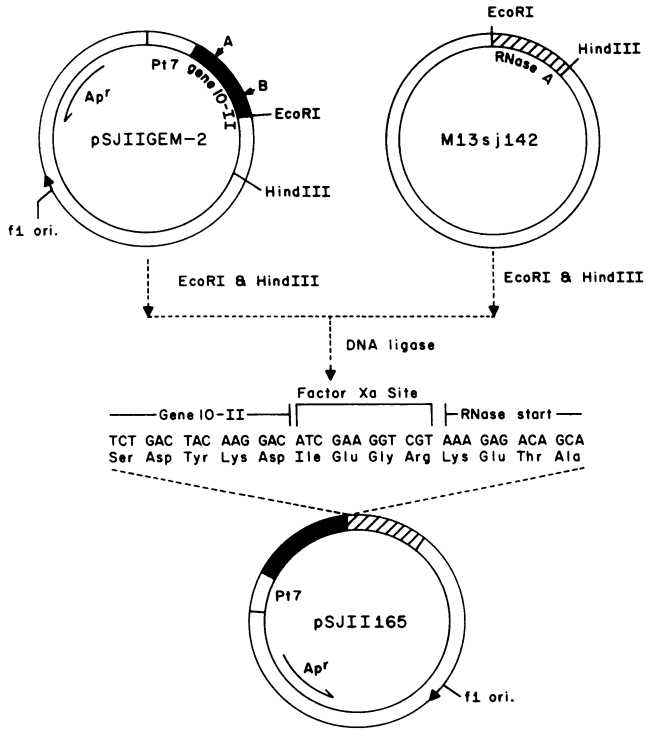

FIG. 1. Construction of the gene 10-II–RNase A fusion protein expression vector. Sites A and B indicate $Arg^{68} \rightarrow Asp^{68}$ and $Arg^{279} \rightarrow Asp^{279}$ mutations, respectively, on the T7 gene 10 protein to create T7 gene 10-II. The factor Xa site is located directly in front of Lys^1 of the RNase A gene. Ap^r, ampicillin resistance; f1 ori., f1 phage origin of replication; Pt7, T7 promoter.

Isolation and Factor Xa Digestion. The resuspended cell pellet was sonicated, jacketed in ice, with 2-sec pulses for 5 min and then centrifuged at 6000 rpm for 45 min. The pellet was washed with 50 ml of 0.5% Triton X-100/1 mM EDTA, centrifuged at 7500 rpm for 1 hr, and then dissolved in 10 ml of 7 M guanidine·HCl/25 mM Tris·HCl/5 mM EDTA, pH 8.0. At this point, the pellet was ≈90% overexpressed fusion protein, as judged by SDS/PAGE. All of the cysteines in this fusion protein were then sulfonated by the procedure of Thannhauser and Scheraga (23). This solution was dialyzed against four changes of factor Xa digestion buffer (50 mM Tris·HCl/0.1 M NaCl, pH 7.0). During the dialysis, most of the sulfonated fusion protein formed a precipitate in the dialysis tube (Fig. 2, lane 2). This precipitated protein solution was brought to 20 ml and digested with factor Xa in two steps. First, about 40–50 μg of factor Xa was added to the suspension and incubated with constant shaking for 14–18 hr at room temperature (Fig. 2, lanes 3 and 4). The supernatant was removed by centrifugation, and sufficient 10 M urea was added to make the supernatant ≈2 M and the pH was reduced to 4.0 with 1 M HCl. This solution was then saved for HPLC purification. Depending on the extent of digestion to this point, the resulting pellet was then resuspended in another 10–15 ml of digestion buffer to which another 10–30 μg of factor Xa was added. This suspension was digested for another 12–16 hr, at which point the digestion of fusion protein was essentially complete (>90%) as judged by SDS/PAGE. Immediately before centrifugation, sufficient 10 M urea and 1 M HCl were added to make the suspension ≈2 M in urea at a pH of 4.0, and to solubilize additional cleaved

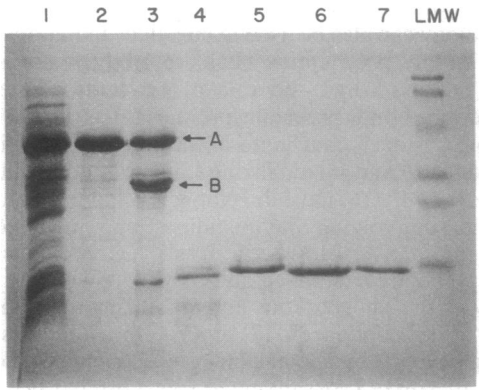

FIG. 2. Reducing SDS/15% PAGE showing the expression-through-purification steps of modified T7 gene 10–RNase A wild-type and mutant fusion proteins. Lanes 1 and 2 represent the whole cell lysate and SO_3-inclusion bodies of the C[40-95]S mutant, respectively. Lanes 3 and 4 show the insoluble and soluble portions, respectively, of the first factor Xa digestion of the gene 10-modified SO_3-C[65-72]S mutant. Arrow A indicates intact (undigested) modified sulfonated fusion protein, and arrow B indicates the portion of the modified sulfonated gene 10 protein remaining after RNase was cleaved off. Lanes 5, 6, and 7 show the sulfonated C[58-110]S mutant after the first Mono S purification (pH 4.0), the folded K41G mutant after the second Mono S purification (pH 7.0), and control native nonrecombinant RNase A, respectively. The slight difference in migration of the RNase bands in lanes 3–7 is due to differences in mutations. LMW, Bio-Rad low molecular weight standard.

RNase A. This supernatant was also saved for HPLC purification.

Purification and Folding. The supernatant of the digestion mixture was purified on an HPLC cation-exchange column (Mono S HR5/5) with 25 mM formic acid/3 M urea at pH 4.0 with a NaCl gradient (Fig. 2, lane 5). The solution of wild-type or mutant RNase A collected from the HPLC column was exchanged into 2 mM EDTA/0.1 M Tris, pH 8.2, by using Pharmacia PD-10 Sephadex G-25M columns, and then incubated in the presence of 1 mM/2 mM (oxidized/reduced) glutathione at 4°C for 24–36 hr. With cyclic cytidine monophosphate (cCMP) as a substrate, the activity of regenerated wild-type SO_3-RNase A was used as a control to monitor the extent of folding. Once folding was judged to be complete, the pH of the recombinant folded protein was then adjusted to 5.0 with glacial acetic acid. This protein was then purified on a Mono S HR5/5 cation-exchange column with 20 mM Hepes at pH 7.0 with an NaCl gradient to remove any partially folded or mixed-disulfide intermediates with glutathione (Fig. 2, lane 6).

Protein Concentration. The concentration of sulfonated unfolded mutant and wild-type RNase A was determined by using an extinction coefficient of 8500 $M^{-1}\cdot cm^{-1}$ at 275 nm (23). The concentration of folded mutant and wild-type protein was determined by using an extinction coefficient of 9800 $M^{-1}\cdot cm^{-1}$ at 277.5 nm (27).

Activity Measurements. The activity of folded wild-type and mutant RNase A was assayed by the method of Crook *et al.* (28), as described by Konishi and Scheraga (29), with the following modifications: the substrate, cCMP, was present in solution prior to the addition of protein, and the change in extinction coefficient at 287 nm was monitored. All measurements were made at 25°C and pH 7.0 unless indicated otherwise.

Spectroscopic Measurements. Absorption spectra were measured on a modified Cary model 14 spectrophotometer (30). CD measurements were made on a modified Cary model 14 spectrometer equipped with a Pockel cell (31). One-dimensional NMR measurements were made on a Unity Varian 500 spectrometer.

Thermal Denaturation. Reversible thermal denaturation was monitored by the change in extinction coefficient of the protein at 287 nm as a function of temperature. The sample temperature was controlled by a Neslab (Portsmouth, NH) RTE-100 thermal water bath, which provided constant circulation through a block holding the sample cuvette. The temperature was determined with a thermistor situated in the reference buffer in the same block.

RESULTS

Expression, Digestion, and Purification. The expression level of fusion protein (≈45 kDa) varied from 50 to 80 mg/liter in LB medium. In general, the yield was higher for mutant RNase A than for wild type. With M9 minimal medium, lower yields were obtained (30–50 mg/liter).

The factor Xa digest was first attempted with solubilized (sulfonated) fusion protein, but the efficiency of cleavage was very poor because of the low solubility of the sulfonated fusion protein. Upon cleavage of the original sulfonated fusion protein with factor Xa, two surprising results were observed. First, the sulfonated fusion protein was digested in two additional places besides the genetically engineered factor Xa site Ile-Glu-Gly-Arg-RNase A (Figs. 1 and 3), reducing the yield and complicating the purification considerably. The two factor Xa "minor sites" in gene 10 were assumed to be Gly^{67}-Arg^{68}-Xaa and Gly^{278}-Arg^{279}-Xaa (32). Therefore, the "minor sites" (A and B in Fig. 1) were removed. The resulting sulfonated fusion protein produced from this expression vector was cleaved significantly only at the engineered factor Xa site (Fig. 2, lanes 3 and 4). Second, digestion of the sulfonated fusion protein as a suspension (50–80 mg in 20 ml) was found to be very efficient. As the digestion progressed, most of the SO_3-RNase A cleavage products became soluble, while the SO_3-gene 10 portion remained insoluble (Fig. 2, lanes 3 and 4). The digestion time was kept to a minimum because factor Xa slowly degraded the cleaved SO_3-RNase A. In addition, the digestions were carried out at pH 7.0 instead of the recommended pH 8.0 (32, 33), to minimize the deamidation of Asn^{67} (23). No significant decrease in factor Xa activity was observed at pH 7.0 over the time period in which the sulfonated fusion protein was digested. Two problems during digestion were coprecipitation of cleaved SO_3-RNase A with SO_3-gene 10-II protein, and proteolytic degradation of cleaved SO_3-RNase A (Fig. 2, lanes 3 and 4). The temperature and incubation time for digestion presented here were optimized to obtain maximum cleavage and solubility of SO_3-RNase A while minimizing degradation. The addition of urea to the suspension after the second digestion solubilized most of the coprecipitated SO_3-RNase A without solubilizing significant amounts of the SO_3-gene 10-II protein.

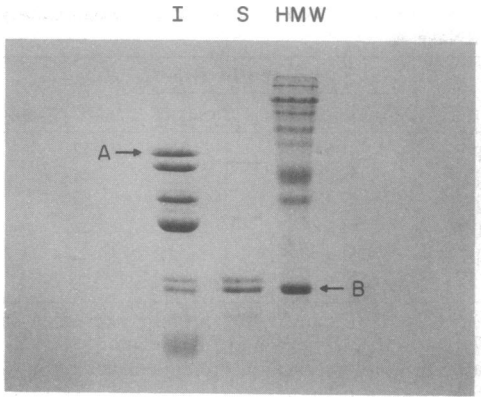

FIG. 3. Reducing SDS/15% PAGE showing factor Xa digest of sulfonated T7 gene 10–RNase A wild-type fusion protein before removal of the two extra cleavage sites. Lanes I and S show soluble and insoluble portions of the digest, respectively. Arrow A indicates the intact sulfonated fusion protein (the other bands in this lane are digestion products). Arrow B indicates the position of wild-type RNase A. The extra band at higher molecular weight in lanes I and S (immediately above that of RNase A) is SO_3-RNase A coupled to a portion of the sulfonated gene 10 protein (from the amino acid following Arg^{279} to its C terminus). HMW, Sigma high molecular weight standard (with added nonrecombinant RNase A).

A Mono S HR5/5 cation exchange column (pH 4.0) with 3 M urea was used for purifying wild-type SO_3-RNase A and all subsequent mutants. The presence of urea prevented precipitation of sulfonated protein on the column. The purity of sulfonated protein after this purification step was >90% as determined by SDS/PAGE (Fig. 2, lane 5). The recovery of sulfonated recombinant protein at this stage was 4–8 mg/liter of cell culture (Table 1). The first 20 amino acids of cleaved wild-type RNase A (both sulfonated and folded) were confirmed by N-terminal amino acid sequencing at the Cornell Biotechnology Center.

Physical-Chemical Characterization. Of the four sulfonated native three-disulfide mutants that were folded, only C[65-72]S and C[40-95]S had partial activity *and* were thermally stable at room temperature (Table 1). For these two mutants, the recovery of folded protein from the Mono S HR5/5 column at pH 7 was 30–40%, in contrast to wild type, whose protein recovery was 80–90%. The lower recovery of folded mutant protein can probably be attributed to the formation of mixed disulfides with glutathione as well as incorrectly folded species. In fact, other minor peaks were observed in the

Table 1. Characteristics of sulfonated and folded wild-type and mutant RNase A

Protein	Yield,* mg/liter		T_m,† °C	Activity‡
	Sulfonated	Folded		
Wild type	4–6	3–5	62	100
C[65-72]S	5–8	1.5–2.5	44	30
C[40-95]S	5–8	1.5–2.5	42	5
C[58-110]S	5–8	—	—	21§
C[26-84]S	5–8	—	—	8§
K41G	5–8	2.5–4	53	0

*Yields per liter of culture for protein expressed in *E. coli* HMS174(DE3)-pLys(S) grown in LB medium.
†Thermal transition temperature. Protein concentration was 10 μM at pH 6.4.
‡Enzymatic activity relative to native nonrecombinant RNase A. Assay temperature was 25°C, unless indicated otherwise.
§Assay temperature was 10°C. Assay was performed before Mono S purification at pH 7.0. Therefore, the activity of purified mutants, each with three native disulfides intact, is probably higher.

chromatograms of these mutants and were chromatographically removed.

The CD spectra (Fig. 4) of purified C[65-72]S and C[40-95]S were quite similar to that of native RNase A and distinctly different from that of SO_3-RNase A. The one-dimensional ^1H NMR spectra of these two mutants at 18.5°C and pH 5.9 (not shown) also indicated some compact structure resembling native RNase A. Specifically, the histidine $C^\varepsilon H$ peaks appeared between 8.3 and 8.8 ppm and were resolved. The aromatic peaks also appeared to be resolved in a native-like pattern (at 6.5 to 7.5 ppm). Since the histidine peaks are very sensitive to their environment and pH, specific peak assignments could not be made with these spectra. However, the histidine and aromatic peaks of both thermally denatured disulfide-intact and SO_3-RNase A nearly coalesced (into one peak for the histidines and three peaks for the aromatics); this coalescence was not observed in the spectra of these mutants (ref. 23; S. Talluri and H.A.S., unpublished observations).

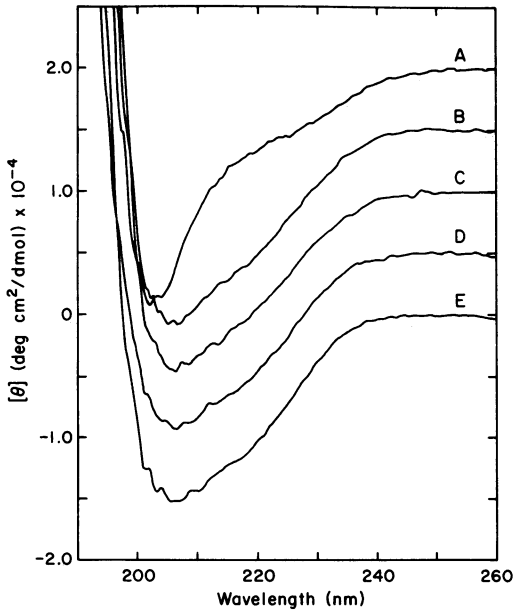

FIG. 4. CD spectra of sulfonated and native wild-type and folded mutant RNase A at 20°C in 10 mM phosphate buffer at pH 7.0 with a protein concentration of 0.03 mg/ml. Spectra: A, SO_3-RNase A; B, C[65-72]S; C, C[40-95]S; D, K41G; E, native wild-type RNase A. In order to display the data clearly, the following constants were added to each ordinate: for spectrum A, 2.0; B, 1.5; C, 1.0; D, 0.5; E, 0 (all $\times 10^4$).

Initially, in refolding buffer at 10°C, 21% and 8% activity relative to native was observed for C[58-110]S and C[26-84]S, respectively, indicating that these mutants probably have a native-like conformation at 10°C. However, this activity was lost when the assay was carried out at 25°C, indicating a low thermal stability for these mutants. Unfortunately, since we were unable to obtain appreciable recovery of folded C[58-110]S and C[26-84]S from the Mono S column (<10% recovery), further physical characterization was not carried out.

Folded K41G was isolated from a Mono S column with >50% recovery and was found to be thermally stable but inactive with cCMP as a substrate (Table 1). The CD spectrum of this mutant was very similar to that of wild-type RNase A (Fig. 4). Finally, the one-dimensional ^1H NMR spectrum of K41G at 18.5°C and pH 5.9 resembled that of folded wild-type RNase A, further indicating a compact folded structure for this mutant.

DISCUSSION

Expression. A versatile expression system for RNase A without modification with an N-terminal methionine has been presented here. Because most of the expressed RNase A-containing fusion protein forms inclusion bodies, this protein is protected from proteolytic degradation. As a result, the yields of all of the RNase A mutants were at least as high as wild type. In fact, the mutants which had significantly lower activity were obtained at higher expression levels (10–20% higher). Even though *E. coli* is largely protected from the toxic effects of overexpressed active RNase A by formation of inclusion bodies, this higher expression level can probably be attributed to lower cytotoxicity of mutant RNase A. In fact, we have observed that RNase A can be refolded quantitatively as an intact fusion protein (unpublished results), indicating that some very low levels of *in vivo* folding might occur in this system.

Initially, protein was expressed in *E. coli* JM109(DE3) which did not contain the pLys(S) plasmid. This strain could not produce consistent levels of expression of wild-type or mutant RNase A fusion protein. This inconsistent expression can probably be attributed to the instability of the expression vector in this strain.

Purification and Factor Xa Digestion. Reversible sulfonation of the isolated fusion protein facilitated the use of a two-step HPLC purification scheme using a high-resolution cation-exchange column at pH 4.0 and pH 7.0 for the sulfonated and folded protein, respectively. In addition, this sulfonated unfolded protein can serve as a very convenient starting point for folding studies with RNase A and for systematically establishing favorable folding conditions for mutants.

Digestion of the relatively insoluble sulfonated fusion protein as a suspension led to very high efficiency of the factor Xa cleavage. For example, 50–80 μg of factor Xa was sufficient to digest 50–80 mg of sulfonated fusion protein (the amount of protein obtained from 1 liter of cell culture) at 90% efficiency. This corresponds to approximately 1:1000 (wt/wt) factor Xa/sulfonated fusion protein digested or 1:100 (wt/wt) factor Xa/purified SO_3-RNase A obtained (Table 1), making this method economical in factor Xa for large-scale protein preparation. In addition, SO_3-RNase A became quite soluble once it was cleaved, and thus was obtained in a relatively pure form even prior to any chromatographic purification (Fig. 2, lane 4).

It was previously reported that the minimum amino acid sequence required for factor Xa recognition and subsequent cleavage is Glu(Asp)-Gly-Arg-Xaa [the complete recognition sequence is Ile-Glu(Asp)-Gly-Arg-Xaa (32)]. Therefore, since the original pGEMEX-2 expression vector did not contain Glu(Asp)-Gly-Arg-Xaa but rather two Gly-Arg se-

quences, both with a hydrophobic amino acid preceding Gly (either Leu^{66} or Val^{277}), this vector was used initially. In contrast to the results obtained by Nagai and Thøgersen (32), the fusion protein obtained from this original pGEMEX-2 expression vector exhibited significant factor Xa cleavage at these sites. In fact, both Gly-Arg-Xaa sites were cleaved competitively with the engineered complete factor Xa site (Fig. 3, lanes I and S). This discrepancy between our results and those reported previously can probably be attributed, at least partially, to the inherent structure dependence of the substrate recognized by factor Xa, especially since the sulfonated fusion protein used here was unfolded and possibly more susceptible to cleavage by factor Xa. Also, variations in the preparations of factor Xa may also be a factor. Finally, the digestion time used here was significantly longer than that used by Nagai and Thøgersen (32). These results demonstrate that fusion proteins of low solubility can be digested with factor Xa with relatively good protein recoveries, but for these proteins the sequence restriction may be more stringent (e.g., no Gly-Arg-Xaa sequences in the fusion protein, especially in the desired conjugate). Fortunately, RNase A contains no Gly-Arg-Xaa sequence; hence, the removal of these "minor sites" from the gene 10 conjugate was entirely adequate.

Physical-Chemical Characterization. The characterization presented here indicates that C[65-72]S, C[40-95]S, and K41G have folded, stable, native-like structures at 25°C, whereas both C[58-110]S and C[26-84]S probably have native-like conformations at lower temperatures. If all four three-disulfide mutants of RNase A do have native-like structure at some temperature, then it can be concluded that no single disulfide bond in RNase A is absolutely necessary to fold RNase A. However, the folding of RNase A is thought to involve multiple pathways (6). Therefore, single disulfide bonds may indeed be necessary in one or more folding pathways of RNase A.

Since both the C[65-72]S and C[40-95]S three-disulfide mutants of RNase A are thermally stable above 30°C at pH 6.4, they are suitable for study by multidimensional NMR to elucidate their structure. The preliminary results in our laboratory suggest that the T_m values of these mutants at pH 4.5 are only 5°C lower (unpublished results) than the T_m values observed at pH 6.4. Low pH and higher temperature greatly improve the resolution achieved in multidimensional NMR studies. Finally, the three-disulfide mutants of RNase A with cysteine replaced by alanine have also been cloned for comparison with the serine mutants.

Since the K41G mutant has no enzymatic activity, the question arises as to whether the absence of activity is due to disruption of structure in the active site, inability of the substrate to bind to the active site, or loss of the required Lys^{41} for catalytic activity. Since glycine is much less bulky than lysine, it seems unlikely that the active site is distorted considerably, although the thermal transition curve of this mutant is quite broad (unpublished data) with a T_m that is 9°C lower than that of native RNase A (Table 1). This difference in thermal transition temperature could also indicate that Lys^{41} has some structural role in the protein. In fact, the x-ray data (34) indicate that Lys^{41} forms a hydrogen bond with Asn^{44}. Also, replacement of the large lysine residue by the smaller glycine would be expected to lead to a change in the free-energy difference between the native and denatured states due to packing effects, even if no specific interactions are involved. However, the studies cited in the Introduction indicate that the most likely cause for the inactivation of RNase A by the Lys → Gly mutation is either the loss of substrate-binding capability or the loss of catalytic function.

In conclusion, we have presented an efficient expression system that can produce large quantities of mutants of RNase A in an unaggregated stable unfolded form. If conformationally stable, these mutants can easily be folded. The preliminary characterization of the disulfide mutants as well as subsequent NMR and other studies to follow should help to elucidate the role that the disulfide bonds play in the folding and structure of RNase A.

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