Crystalline semisynthetic ribonuclease-S’

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ABSTRACT Crystals of solid phase-derived semisynthetic ribonuclease-S’ were prepared and compared with those for native ribonuclease-S’ and -S. The semisynthetic species used was the noncovalent complex of synthetic fragment (1–20), corresponding to residues 1 through 20 of bovine pancreatic ribonuclease-A (ribonuclei 3’-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22), and native ribonuclease-S(21–124), the fragment containing residues 21 through 124 of ribonuclease-A. This semisynthetic complex was completely active enzymatically, was homogeneous as judged by polyacrylamide gel electrophoresis, and had no greater than trace amounts of excess ribonuclease-S(21–124) as judged by affinity chromatography. Crystallization of both semisynthetic and native ribonuclease-S’ at pH 5.3 resulted in well-formed crystals with the symmetry of space group P3121 and unit cell dimensions $a = b = 44.52, c = 97.3$ A. This crystal form corresponds to the $Y$ form of native ribonuclease-S previously reported (Wyckoff et al. (1967) J. Biol. Chem. 242, 3749–3753). X-ray diffraction patterns of the crystals were indistinguishable, indicative of the structural identity of semisynthetic and native ribonuclease-S’.

The ability to prepare synthetic proteins with a planned chemical mutation of important residues is of potential importance in the elucidation and engineering of conformational and functional features of proteins. Obtaining such species generally is limited by technical problems encountered in the synthesis and purification of large polypeptides to a state which will allow detailed characterization. One approach to circumvent these difficulties is to synthesize peptide fragments which can combine integrally with complementary native fragments to yield semisynthetic protein complexes (1–6). In this way, synthesis can be carried out for relatively small peptides and the products obtained allow the direct study of proteins.

We previously (7, 8) have prepared a semisynthetic ribonuclease-S’ (RNase-S’), a noncovalent complex containing solid phase-derived synthetic fragment (1–15), corresponding to residues 1 through 15 of bovine pancreatic ribonuclease-A (ribonuclei 3’-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22) and the native fragment containing residues 21 through 124 of [native ribonuclease-S, RNase-S(21–124)]. This semisynthetic species has been purified to a state of high enzymic activity and has been used in detailed studies of both conformational and functional features of native RNase-S by the preparation of analogues (7, 10, 11). These studies have increasingly emphasized the desirability of being able to elucidate detailed structural features of semisynthetic analogues. One method useful for achieving such a goal is x-ray diffraction analysis of crystalline protein. The present study was carried out to demonstrate the ability to prepare crystals of normal sequence SRNase-S’ and to compare the x-ray diffraction patterns for such crystals with those for native ribonuclease-S’ (RNase-S’).
at pH 5.3; solution 3, 75% ammonium sulfate in 0.1 M sodium acetate at pH 5.5; solution 4, 75 mg/ml of RNase complex in solution 1. For a particular trial, 25 μl of solution 4, followed by 25 μl of solution 2, were placed in the flattened well of a siliconized culture slide (Arthur H. Thomas Co., well dimensions 15 mm diameter and 3 mm depth). After the solutions were mixed, the well was closed off with a small glass cover and sealed with silicone grease. After 2-3 days at room temperature, large crystals of approximate dimensions 0.3-0.7 mm were obtained. At this stage, the liquid of crystallization was replaced with solution 3. This was done in five steps; in each, about 80% of the mother liquor was withdrawn and replaced with 25 μl of solution 3. This procedure obviated the need for physically transferring the crystals, thus avoiding damage.

Alternate crystallization trials were performed at pH 5.9 and 6.1.

**X-Ray Diffraction Analyses.** Crystals were mounted wet in siliconized glass capillaries. Diffraction patterns were recorded with a Supper precession camera using CuKα radiation. Lattice parameters were determined from 15° precession photographs with a Supper Circular Film Measuring Device.

**RESULTS**

The SRNase-S' obtained in this study was completely active by comparison with native RNase-A and RNase-S'. The RNase-A standard used had specific enzymic activity of 20.8 ΔA287 nm/min per μmol versus a value of 20.4 for RNase-S'. By comparison, SRNase-S' had a specific activity of 19.7 ΔA287 nm/min per μmol.

The gel electrophoretic analysis of the semisynthetic complex was carried out by comparison with native RNase-S'. The resulting gels, shown in Fig. 1, indicate the electrophoretic homogeneity of SRNase-S'.

As a further check of homogeneity, SRNase-S' was chromatographed on Sepharose-5'-[4-aminophenylphosphoryl]-uridine-2'(3')-phosphate under competitive elution conditions (see Materials and Methods). The results indicate the presence of a trace amount of unretarded material and the elution of the semisynthetic complex in one peak with the same position found for native RNase-A and -S. Material in the unretarded peak showed enzyme activity upon addition of excess RNase-S' (1-20); this activity indicates the presence of a small amount of excess RNase-S (21-124) (<5%) in the SRNase-S' preparation obtained from sulfoethyl-Sephadex chromatography.

SRNase-S' was dissociated into component fragments by Sephadex G-75 fractionation in 50% acetic acid (9). The recovered synthetic-(1-20) peptide gave an amino acid composition which is in close agreement (±6%) with that obtained with RNase-S (1-20).

**Crystallography of SRNase-S'**

When SRNase-S' was crystallized at pH 5.3, several large and many small crystals were obtained. A representative photograph of some of these crystals is shown in Fig. 2. The crystals are well formed and are excellent diffraectors. Precession photographs of various reciprocal lattice nets reveal symmetry indicative of space group P3₁21. The lattice dimensions are a = b = 44.82 ± 0.04, c = 97.3 ± 0.1 Å. These clearly correspond to the Y form of RNase-S previously reported by Wyckoff et al. (18). The crystals of RNase-S' obtained here at pH 5.3 also were of the Y form, with identical unit cell dimensions. The x-ray diffraction patterns of the Y-form crystals of SRNase-S' and RNase-S' were found to be indistinguishable (see, for example, Fig. 3).

![Polyacrylamide gels of semisynthetic and native RNase-S'. The arrows indicate the electrophoresis front, determined with tracking dye and emphasized by insertion of metal bars after electrophoresis.](attachment:polyacrylamide_gels.png)

Crystals of the Y form also could be obtained for SRNase-S' at pH 6.1. However, in this study, crystallization of native RNase-S', and -S, at pH 6.1 most often led to only small crystals of the Y form but large crystals of a second form. This latter form, found to possess the symmetry of the same space group (P3₁21) but with unit cell dimensions a = b = 65.33 ± 0.04, c = 65.08 ± 0.03 Å, is the W form also noted previously for native RNase-S (18). (One specific attempt to obtain W-form crystals for SRNase-S' was unsuccessful.) The crystals of the different native complexes obtained in the W form also gave indistinguishable diffraction patterns.

Washed Y-form crystals of SRNase-S' were redissolved and assayed enzymatically in three separate trials. Specific enzymic activities of 12, 21, and 34 ΔA287 nm/min per μmol were measured in these trials. Although there is considerable variation in these values, apparently due to inaccuracies incurred in the handling of very small amounts of redissolved protein, the significant activities measured (average = 22 ΔA287 nm/min per μmol) indicate an essentially full retention of enzymatic activity during crystallization.
DISCUSSION

We have been able to obtain crystals of normal-sequence SRNase-S' of a morphology, corresponding to the Y form of RNase-S (18), which is the same as that of crystals obtained in this study for native RNase-S'. For native RNase-S, the Y form has been solved by x-ray diffraction analysis to high resolution (18, 19). The semisynthetic complex crystals, which are obtained most reproducibly at pH 5.3, were found to be excellent diffractors, amenable to high resolution x-ray analysis. Further, the crystals appear to be quite resistant to radiation damage.

Comparative x-ray diffraction analysis of Y-form crystals of SRNase-S' and native RNase-S' have shown no observable differences in the diffraction patterns obtained (as in Fig. 3). This indicates that these species are isomorphous. This isomorphism indicates that SRNase-S' has a detailed three-dimensional conformation identical with that of the native complex.

Our data reinforce the contention, which we have made previously from data in solution (8, 20), that SRNase-S', of a quality needed for detailed protein characterization, can be obtained via the solid phase synthesis route. It now appears possible, for important analogues of SRNase-S' made by amino acid replacement or deletion, to study structural properties by x-ray diffraction analysis, provided that crystals of such complexes can be obtained. Correlation of these structures with the known structure of native RNase-S could be particularly useful in understanding ribonuclease conformation and function.

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