Preparation and Crystallization of 41-Dinitrophenyl Ribonuclease S*

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ABSTRACT: The partial digestion of 41-dinitrophenyl ribonuclease A, an enzymatically inactive derivative of bovine pancreatic ribonuclease A, by the bacterial protease subtilopeptidase A results in the formation of a doubly modified derivative in which the peptide bond between residues 20 and 21 has been hydrolyzed. As the reaction is analogous to that observed with unmodified ribonuclease A and subtilisin to form ribonuclease S (Richards, F. M., and Vithayathil, P. J., *J. Biol. Chem. 234*, 1459 (1959)), the derivative has been named 41-dinitrophenyl ribonuclease S. No recovery of enzymatic activity results from this modification. The space group of crystals of 41-dinitrophenyl ribonuclease S is the same as that of ribonuclease S crystals grown under the same conditions and the unit cell dimensions of the two types of crystals are nearly identical. The dissociation constant at pH 5.5 of the N-terminal peptide (residues 1–20 or the S-peptide fraction) and the remainder of the molecule (residues 21–124 or the 41-dinitrophenyl S-protein fraction) is $3 \pm 2 \times 10^{-6}$ M, a value which does not differ significantly from the dissociation constant measured by us for the analogous S-peptide and S-protein portions of ribonuclease S. During the course of preparing 41-dinitrophenyl ribonuclease S a method of purification of 41-dinitrophenyl ribonuclease A was developed which results in a preparation of this derivative exhibiting <0.001% of the activity of ribonuclease A.

The three-dimensional conformation in the crystal of bovine pancreatic RNase A[1] and of bovine pancreatic RNase S is now known to a resolution of 2 Å (Kartha *et al.*, 1967; Wyckoff *et al.*, 1970). Analogous information for crystals of derivatives of these proteins which exhibit modified enzymatic activity should reveal the nature and degree of the structural modification responsible for the perturbed catalytic activity. In this way it may be possible eventually to delineate the maximum structural flexibility consonant with a given magnitude of substrate binding or rate of substrate conversion.

The preparation and characterization of 41-DNP RNase S, a doubly modified derivative of bovine pancreatic RNase A which exhibits severely reduced, possibly nil, enzymatic activity, is reported here. Crystals of this derivative have been prepared and have been found to be sufficiently isomorphous with crystals of RNase S to permit an examination of the structural differences which are evident at a resolution of 3 Å between these two proteins (Allewell, 1969).

Materials and Methods

Bovine pancreatic RNase A, lots RAF9DA and RAF9FA, was a salt-free, lyophilized preparation purchased from Worthington Biochemical Corp.

41-DNP RNase A was prepared by the method of Hirs *et al.* (1965). Initial fractionation of the reaction mixture by chromatography on IRC-50 (Bio-Rex 70, − 400 mesh) gave poorer resolution than that described by these authors, who used a resin preparation which had been fractionated by the flotation method of Hamilton (1958). Satisfactory purification of the derivative was achieved, however, by rechromatography at room temperature in an IRC-50–NaCl system (Crestfield *et al.*, 1963; Heinrikson, 1966). A 2.2 × 35 cm column of IRC-50 which had been equilibrated with 0.2 M sodium phosphate (pH 6.47) was flushed with 100 ml of 0.266 *m* NaCl at a rate of 15 ml/hr. The sample (30–60 mg) was dissolved in 0.266 *m* NaCl (3–5 ml), the pH was adjusted to a value of 6.40–6.45, and the solution was held at 65° for 10 min (Crestfield *et al.*, 1962). The cool solution was applied immediately after the 100-ml portion of 0.266 *m* NaCl had passed through the column. Elution was effected with 0.266 *m* NaCl at a rate of 15 ml/hr. The column was reequilibrated with 0.2 M sodium phosphate (pH 6.47) prior to each use.

Subtilopeptidase A, marketed under the name of Protease VIII, was a lyophilized powder of unknown specific activity purchased from Sigma Chemical Co.

Partial Digestion of 41-DNP RNase A by Subtilopeptidase A. ANALYTICAL SCALE. A 500-μl portion of a 2% solution of lyophilized, salt-free 41-DNP RNase A in 0.1 M Tris-HCl (pH 8.0) was cooled to 5°. A 10-μl portion of a freshly prepared 0.1% solution of subtilopeptidase A in 0.1 M Tris-HCl (pH 8.0) was added to give a 41-DNP RNase A:subtilopeptidase A weight ratio of 1000. The solution was held at 5°. At appropriate times 50-μl aliquot samples (1 mg of protein) were removed, diluted with 500 μl of 0.04 N HCl (to give a pH of 2–3), and held 1 hr at 23°. At this pH and temperature-irreversible denaturation of the subtilopeptidase A rapidly occurred (Johansen and Ottesen, 1964). A 250-μl portion of 0.2 M sodium phosphate (pH 6.47) was then added, the solution was cooled to 5°, and was applied quantitatively to a 1.8 × 25 cm column of IRC-50 which had been equilibrated with 0.2 M sodium phosphate (pH 6.47). The column was thermostated at 5°. Elution was effected by pumping 0.2 M sodium phosphate (pH 6.47) through the column at 17 ml/hr. The effluent was monitored at 220 nm by the use of a flow cell of 10-mm path length and a Beckman DB spectrophotometer. The absorbance was recorded with a Sargent Model SRL recorder. Calibration of the system with standard solutions of RNase A and 41-DNP RNase A revealed a direct proportionality between the amount of each species applied and the area of the respective effluent envelopes.

PREPARATIVE SCALE. The composition of the digestion

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[1] The nonstandard abbreviations used are: RNase A, ribonuclease A; RNase S, ribonuclease S.

mixture was exactly as described for the analytical scale digestions. The amount of 41-DNP RNase A used in particular preparations varied between 30 and 70 mg. The solution was held at 5° for 18 hr. At the end of this time the pH of the cold, stirred solution was lowered to 3.0 by the dropwise addition of 1 N HCl. After 1 hr at 23° the pH of the stirred solution was raised to 6.4 by the dropwise addition of 1 N ammonium hydroxide. The solution was applied to a 2.2 × 35 cm column of IRC-50 which had been equilibrated at 5° with 0.2 M sodium phosphate (pH 6.47). Elution was effected at 5° with 0.2 M sodium phosphate (pH 6.47). The fractions which contained the major chromatographic component were lyophilized, desalted by dialysis in acetylated tape (Craig and Konigsberg, 1961) against water at 5°, and again lyophilized. The resulting material was stored at −20°.

Fractionation of the Major Chromatographic Component by Gel Filtration in 50% Acetic Acid. The salt-free, lyophilized powder (20–40 mg) was dissolved in 2–3 ml of water and an equal volume of glacial acetic acid was added to the stirred solution. The solution was applied to a 1.9 × 75 cm column of Sephadex G-75 (coarse grade, bead form) which had been equilibrated with 50% acetic acid. Elution was effected with 50% acetic acid at 23° at 15 ml/hr. A 5- or 10-μl sample of each effluent fraction (1.2–1.5 ml) was taken to dryness in a polypropylene tube, subjected to alkaline hydrolysis, and allowed to react with ninhydrin reagent (Hirs, 1967). With the yellow fractions the absorbance at 420 nm was obtained directly by the use of a 1-ml cuvet. The fractions containing the two bands of ninhydrin-positive material were, respectively, pooled, diluted tenfold with water, and lyophilized. The resulting powders were stored at −20°. In more recent preparations the 50% acetic acid has been replaced by 5% formic acid (Hearn *et al.*, 1971). The quality of the fractionation is identical and it is no longer necessary to dilute the fractions prior to lyophilization.

The N-terminal residues of the 41-DNP S-protein fraction were determined by the cyanate method (Stark and Smyth, 1963; Stark, 1967).

The dissociation constant of the S-peptide and 41-DNP S-protein portions of 41-DNP RNase S was determined from the ultraviolet difference spectrum which was generated upon the recombination of the two chains at neutral pH (*cf.* Richards and Logue, 1962; Woodfin and Massey, 1968). Stock solutions of 41-DNP S-protein were prepared by dialysis at 5° of a concentrated solution in acetylated tape against 0.001 M sodium phosphate (pH 5.48). Stock solutions of S-peptide were prepared by direct dissolution of a lyophilized preparation in 0.001 M sodium phosphate (pH 5.48). The pH of these latter solutions was readjusted to 5.48 from a value of approximately 4.5 by the addition of approximately 10 μl of 1 N NaOH. This pH adjustment resulted in a 0.002 M sodium acetate concentration in the S-peptide solutions. All stock solutions were standardized by amino acid analysis of 22-hr hydrolysates of aliquot samples. Difference spectra were obtained at 23° with a Cary Model 15 recording spectrophotometer and double-sector Yankeelov cuvets (Yankeelov, 1963) (Pyrocell Manufacturing Co.). A 900-μl sample of the 41-DNP S-protein solution was placed in one of the compartments of both the reference and sample cuvets. Aliquot samples (10–700 μl) of the S-peptide stock solution were placed in the second compartment of both cuvets and the total volumes in these latter compartments were brought to 900 μl by the addition of a suitable volume of 0.001 M sodium phosphate (pH 5.48). The contents of these latter compartments were carefully mixed with a small polyethylene paddle. All samples were delivered from calibrated Lang–Levy micropipets and the accuracy of the volumes delivered was checked by weighing the cuvets after each addition. After the recording of the base-line spectrum the solutions in the two compartments of the sample cuvet were mixed. The removal and replacement of the sample cuvet in its holder was sufficient to cause a significant change in the base-line spectrum (as much as 0.01 *A* unit). The mixing was achieved, therefore, with the cuvet in place by the repeated withdrawal and redelivery of a mixture of portions of both solutions. A 500-μl Lang–Levy micropipet fitted with a 2-cm section of narrow-bore polyethylene tubing (Intramedic Polyethylene Tubing PE 160, Clay-Adams, Inc.) was used for this purpose. The removal of solution from a compartment followed by redelivery back to the same compartment caused no detectable change in the base-line spectrum. When solutions were actually mixed the completion of the process was determined by the cessation of change in the difference spectrum.

Crystallization of 41-DNP RNase S. A 10-mg sample of lyophilized salt-free 41-DNP RNase S was dissolved in 250 μl of 6.0 M cesium chloride contained in a 12 × 35 mm screw-top vial. A 50-μl aliquot of 2 M potassium phosphate (pH 5.5) and a 200-μl aliquot of 80% saturated ammonium sulfate were added and the solutions were mixed by inversion. A substantial amorphous precipitate was formed at this time. Initially the mixtures were seeded with fragments of RNase S crystals; after they became available fragments of 41-DNP RNase S crystals were used. Prior to their use for diffraction studies the crystals were nominally freed of cesium chloride by equilibration with a solution composed of saturated ammonium sulfate (65 ml), 2 M potassium phosphate (pH 5.5) (10 ml), and water (25 ml).

Enzymatic activity was measured as described by Murdock *et al.* (1966) with cytidine 2′,3′-cyclic phosphate (Schwarz–Mann) as the substrate.

Acid hydrolysates were prepared with twice-distilled, constant-boiling HCl in evacuated tubes at 110°.

Amino acid compositions were determined with a Beckman 120C amino acid analyzer.

Results and Discussion

Chromatography of 41-DNP RNase A Preparations in an IRC-50–NaCl System. The effluent profile obtained from the chromatography of a 41-DNP RNase A preparation in an IRC-50–NaCl system is shown in Figure 1. The preparation had been obtained by the chromatography on IRC-50 (0.2 M sodium phosphate, pH 6.47) of the products of the reaction at pH 8.0 between RNase A and dinitrofluorobenzene (Hirs *et al.*, 1965) and it exhibited 21% of the specific activity of RNase A toward cytidine 2′,3′-cyclic phosphate. In the rechromatography (Figure 1) the band of yellow protein which emerged between fractions 50 and 65 had no detectable activity against this substrate in an assay system where 0.001% of the specific activity of RNase A was readily detectable. No absorbance at 278 nm was detected in fractions 70–220. Conversion at fraction 200 to an eluting buffer composed of 0.2 M sodium phosphate (pH 6.47) caused the prompt emergence of two bands of protein, both of which exhibited 100% of the specific activity of RNase A against cytidine 2′,3′-cyclic phosphate. The nature of these proteins was not investigated further.

Crestfield *et al.* (1963) successfully resolved 1-carboxymethylhistidine-119–RNase A, 3-carboxymethylhisitidine-12–RNase A, and RNase A with an IRC-50–NaCl system very

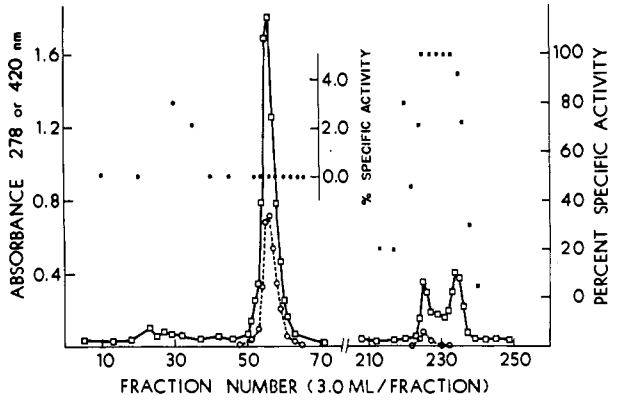

FIGURE 1: Chromatography of a 41-DNP RNase A preparation in an IRC-50–NaCl system. Absorbance at 278 nm (□) and at 420 nm (○). Specific activity against cytidine 2′,3′-cyclic phosphate of selected fractions between 10 and 66 (●), and of selected fractions between 213 and 240 (■). Column (2.2 × 35 cm) had been equilibrated with 0.2 M sodium phosphate (pH 6.47); just prior to sample application it was flushed with 100 ml of 0.266 *m* NaCl at a rate of 15 ml/hr. Elution was effected up to fraction 200 with 0.266 *m* NaCl; at this time the eluting buffer was changed to 0.2 M sodium phosphate (pH 6.47). Sample size, 38 mg; flow rate, 18 ml/hr; temperature, 23°. Recovery of applied absorbance (278 nm) at fraction 250 was 94%.

similar to the one used by ourselves. The order of elution of these three proteins differed from their order in the IRC-50–0.2 M sodium phosphate (pH 6.47) system; the authors found the changed behavior to be compatible with the hypothesis that chromatographic position depended in part upon phosphate binding and that such binding was diminished in the two carboxymethylated derivatives. Our observation of the apparently quantitative separation of 41-DNP RNase A from enzymatically active contaminants by the IRC-50–NaCl system may also reflect reduced binding of inorganic phosphate by the derivative as compared to the magnitude of binding by the active contaminants. A measurement of the binding of inorganic phosphate to 41-DNP RNase A is not available; however, the dissociation constant of cytidine 3′-phosphate and 41-DNP RNase A is 2.2×10^{-4} M at pH 5.5 whereas the corresponding constant for RNase A is 1.2×10^{-5} M (Ettinger and Hirs, 1968).

Partial Digestion of 41-DNP RNase A by Subtilopeptidase A. The effluent profile obtained from the chromatography on IRC-50 of the products of the partial digestion of 41-DNP RNase A by subtilopeptidase A is shown in Figure 2. The chromatographic position of the major component (fractions 56–70) bears the same relationship to the position of 41-DNP RNase A (arrow) as does the position of RNase S to that of RNase A when these proteins are chromatographed in the same system (see, for example, Doscher and Hirs (1967), Figure 1).

The amino acid composition of the major component was indistinguishable from that of 41-DNP RNase A indicating that no portion of the polypeptide chain had been lost as a result of the exposure to subtilopeptidase A.

With 41-DNP RNase A preparations which had a specific activity of <0.001% the specific activity of the major component was also <0.001%; when the 41-DNP RNase A preparation exhibited detectable activity, *e.g.*, 2.4%, the activity of the major component was very similar, *viz.*, 2.5%. A probable basis for this observation is the similar susceptibility of the active contaminants in the preparations to partial proteolysis by subtilopeptidase A.

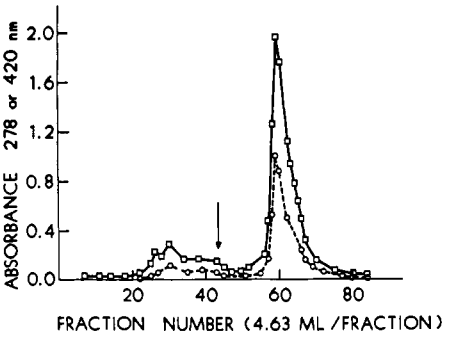

FIGURE 2: Chromatography on IRC-50 of the products of the partial digestion of 41-DNP RNase A by subtilopeptidase A. Absorbance at 278 nm (□) and at 420 nm (○). Column, 2.2 × 35 cm; eluting buffer, 0.2 M sodium phosphate (pH 6.47); sample size, 55 mg; flow rate, 28 ml/hr; temperature, 5°. Arrow denotes the position of 41-DNP RNase A on this column. Recovery of applied absorbance (278 nm) at fraction 80 was 88%.

Characterization of the Major Component from the Partial Digestion of 41-DNP RNase A by Subtilopeptidase A. The filtration of the major component through Sephadex G-75 under acidic conditions, *e.g.*, 50% acetic acid, caused the separation of the material into two bands, A and B (Figure 3). The amino acid compositions of the materials in these two bands correspond to those for residues 21–124 (band A) and residues 1–20 (band B) (Table I). The catalysis by subtilopeptidase A of the hydrolysis of the peptide bond between residues 20 and 21 of 41-DNP RNase A would result in the formation of the polypeptides found in bands A and B. This susceptibility to the action of subtilopeptidase A is qualitatively analogous to that observed for RNase A (Richards and Vithayathil, 1959). Consequently, the major component in the digest has been designated 41-DNP RNase S and bands A and B as the 41-DNP S-protein fraction and the S-peptide fraction, respectively. It is noteworthy that the material in the 41-DNP S-protein fraction absorbs 420-nm radiation whereas the material in the S-peptide fraction does not. This observation is consistent with the assignment of the dinitrophenylation exclusively to lysine-41 in the original derivative (Hirs and Kycia, 1965) and with the identification of the material in

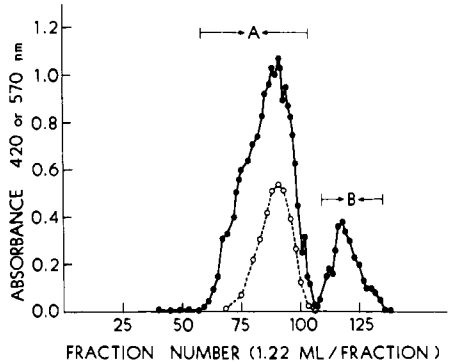

FIGURE 3: Fractionation by filtration through Sephadex G-75 of the major component from the partial digestion of 41-DNP RNase A by subtilopeptidase A. Absorbances at 570 nm (●) are the ninhydrin color values obtained after alkaline hydrolysis of 10-μl samples from the fractions. Absorbances at 420 nm (○) are direct readings of the fractions. Column, 1.9 × 75 cm; eluting solvent, 50% acetic acid; sample size, 20 mg; flow rate, 15 ml/hr; temperature, 23°. Effluent bands were pooled into fractions A and B as indicated.

TABLE I: Amino Acid Compositions[a] of Bands A and B from the Fractionation by Filtration through Sephadex G-75 of the Major Component from the Partial Digestion of 41-DNP RNase A by Subtilopeptidase A.

Amino Acid	Band A	Residues 21–124 of RNase A[b]	Band B	Residues 1–20 of RNase A[b]
Lysine	6.88[c]	8	2.00	2
Histidine	3.01	3	1.00	1
Arginine	3.08	3	1.01	1
Aspartic acid	14.1	14	1.04	1
Threonine	7.65	8	1.96	2
Serine	11.8	12	3.05	3
Glutamic acid	9.07	9	2.98	3
Proline	4.13	4		
Glycine	3.41	3	0.16	
Alanine	7.44	8	5.03	5
Half-cystine	5.62	8		
Valine	8.90	9		
Methionine	2.68	3	0.92	1
Isoleucine	2.06	3		
Leucine	1.99	2		
Tyrosine	5.46	6		
Phenylalanine	1.95	2	0.98	1

[a] Values were determined after 22-hr hydrolysis and are given as molar ratios. Threonine and serine values have been corrected for 5 and 10% hydrolytic destruction, respectively. No other corrections for hydrolytic losses or incomplete hydrolysis have been applied. Values below 0.04 residue are not included. [b] Smyth *et al.* (1963). [c] Seven lysine residues are to be expected in the hydrolysate from 41-DNP S-protein as the ε-DNP-lysyl residue at position 41 is not converted to lysine under the hydrolysis conditions (Hirs *et al.*, 1965).

TABLE II: N-Terminal Residues of S-Protein and of 41-DNP S-Protein.

End Group	Residue/Mole[a] S-Protein	41-DNP S-Protein
Lysine	0.01	0.02
Histidine	0.02	0.07
Arginine	0.05	0.13
Aspartic acid or asparagine	0.05	0.03
Threonine	0.05	0.05
Serine	0.81	0.91
Glutamic acid or glutamine	0.13	0.07
Glycine	0.06	0.07
Alanine	0.02	0.03
Half-cystine	0.07	0.08

[a] Values have been corrected on the basis of the recoveries reported by Stark and Smyth (1963).

band A as residues 21–124 of the original derivative. The basis for the variation in the ratio of the absorbance at 420 nm to that at 570 nm in this band is not evident.

A derivative in which there had occurred hydrolysis of peptide bonds at positions interior to the two outermost disulfide bonds might exhibit all the properties so far described. Consequently, the possibility of additional points of hydrolysis was investigated by the quantitative determination of the N-terminal residues in the 41-DNP S-protein fraction. Application of the cyanate procedure (Stark and Smyth, 1963; Stark, 1967) demonstrated the presence of minor amounts of several N-terminal residues in addition to the serine N-terminal residue arising primarily from the hydrolysis of the peptide bond between residues 20 and 21 (Table II). None of the residues other than serine was present in an amount greater than 0.13 mole/mole of 41-DNP S-protein. Similar amounts of the same types of residue were detected in the S-protein fraction derived from the subtilopeptidase digestion of unmodified RNase A (Table II). No attempt was made to eliminate artifactual N-terminal residues by running a protein blank (Stark, 1967). Previous reports have noted the presence of minor amounts of a number of N-terminal residues in subtilopeptidase digestion mixtures of RNase A (Richards and Vithayathil, 1959; Markus *et al.*, 1968). There may be little or no relationship between our observations and those quoted, however. In the earlier studies there exist two distinct sources of additional N-terminal residues: they may arise from small peptides derived from extensive digestion of a minute portion of the RNase S (or RNase A) (Richards, 1955) or they may reflect the hydrolysis of one or two additional bonds in the S-protein moiety of a minor portion of the RNase S. Chromatographic fractionation has presumably eliminated the former source in our experiments.

Chromatographic examination of the S-peptide fraction from RNase S (resulting from subtilopeptidase rather than Nagarse digestion) has revealed the presence of minor peptide components which are probably the peptide composed of residues 1–21 and the methionine-13 sulfoxide derivatives thereof (Doscher and Hirs, 1967). The analogous examination of the S-peptide fraction from 41-DNP RNase S has not yet been performed.

Dissociation Constant of 41-DNP RNase S. The recombination at pH 5.48 of the S-peptide and 41-DNP S-protein fractions generated a complex difference spectrum in the ultraviolet range (Figure 4). The spectrum between 275 and 300 nm is qualitatively very similar to that generated upon the recombination of S-peptide and S-protein (Richards and Logue, 1962; Woodfin and Massey, 1968). At longer wavelengths, *i.e.*, 310–360 nm, a broad band is observed with the dinitrophenylated S-protein which is not seen with the unmodified S-protein.

The dependence of the absorbance difference generated at 287 nm upon the relative concentrations of the S-peptide and the 41-DNP S-protein fractions was utilized to calculate a dissociation constant for 41-DNP RNase S (*cf.* Woodfin and Massey, 1968). This procedure relies upon the assumption that the magnitude of the absorbance difference bears a constant relationship to the concentration of 41-DNP RNase S. The molar absorptivity change at 287 nm for the generation of 41-DNP RNase S was determined by the extrapolation to the ordinate of a plot of $1/\Delta_{287\ nm}$ *vs.* 1/(S-peptide) for molar ratios of S-peptide to 41-DNP S-protein between 2 and 7. This value was found to be 1660 ± 70 M^{-1} cm^{-1}, which is a markedly larger increase than the values of 900–1000 M^{-1} cm^{-1} previously reported for RNase S (Sherwood and Potts, 1965;

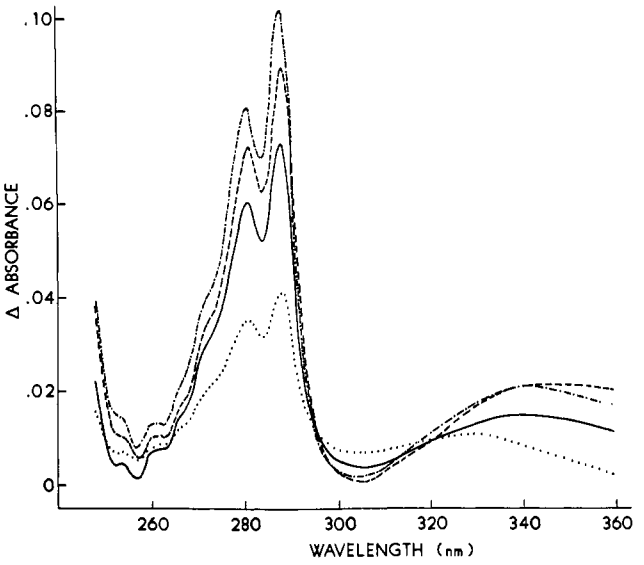

FIGURE 4: Ultraviolet difference spectra generated upon the recombination of the 41-DNP S-protein fraction and the S-peptide fraction in 0.001 M sodium phosphate (pH 5.48). Concentration of the 41-DNP S-protein fraction in the samples, 6.51×10^{-5} M. Mole ratio of the S-peptide fraction to the 41-DNP S-protein fraction, 0.400, · · · ·; 0.808, ——; 2.39, – – – –; and 7.21, –·–·–·.

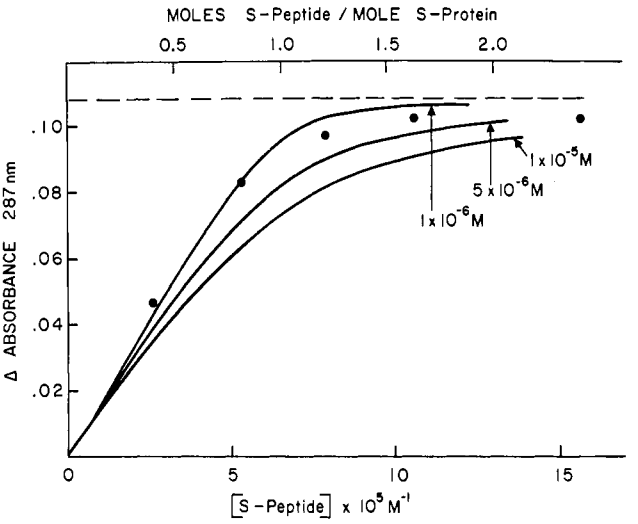

FIGURE 5: Dissociation constant for 41-DNP RNase S in 0.001 M sodium phosphate (pH 5.48). Absorbance changes are given for a cuvet path length of 10 mm.

Woodfin and Massey, 1968) or the value of 1110 ± 25 M^{-1} cm^{-1} measured by ourselves for RNase S during the course of the present work. Such an enhanced change in the absorbance at this wavelength may be the result of a contribution from the dinitrophenyl chromophore; if so, the environment of this residue must be altered upon the binding of the S-peptide. Ettinger and Hirs (1968) have concluded from solvent perturbation studies of 41-DNP RNase A that the dinitrophenyl moiety in this derivative is partially folded into the protein matrix. Alternatively, the modification of the environment of one or more of the six tyrosine residues in 41-DNP RNase S *vis-a-vis* RNase S and/or in the 41-DNP S-protein *vis-a-vis* the S-protein are also possible bases for the relative increase in this value.

The dissociation constant for 41-DNP RNase S was calculated to be $3 \pm 2 \times 10^{-6}$ M (Figure 5). This value is significantly smaller than the dissociation constant of 7×10^{-5} M previously reported for RNase S under very similar conditions (Woodfin and Massey, 1968), but it does not differ, within experimental error, from the value of $7 \pm 3 \times 10^{-6}$ M measured for RNase S in this laboratory. The basis for the discrepancy in the two values for RNase S is not obvious. We measured S-peptide and S-protein concentrations by amino acid analysis whereas Woodfin and Massey utilized the extinction coefficient reported by Potts *et al.* (1964) for S-protein solutions and the ninhydrin color obtained after alkaline hydrolysis for S-peptide solutions. We observed 60% of the light scattering encountered by Woodfin and Massey; we corrected for it by extrapolation to 287 nm of absorbance values observed in the wavelength range of 300–350 nm (Leach and Scheraga, 1960) whereas Woodfin and Massey based their corrections upon the absorbance values observed at 262 nm.

Several lines of evidence suggest that a conformation change occurs upon the dinitrophenylation of lysine-41 in RNase A (Murdock *et al.*, 1966; Ettinger and Hirs, 1968; Allewell, 1969). The absence of a significant change in the dissociation constant of the S-peptide and S-protein moieties as a result of this reaction is, therefore, somewhat surprising.

Rates of Digestion of RNase A and 41-DNP RNase A by Subtilopeptidase A. In view of the identity of the major site of initial digestion by subtilopeptidase A in RNase A and in 41-DNP RNase A a study of the rates of this reaction in the two proteins was undertaken. In Figure 6 are shown their respective rates of disappearance from digestion mixtures of otherwise identical composition. The time course for the two proteins is clearly different; whether this difference reflects a structural difference between RNase A and 41-DNP RNase A or one between substances which appear at a later stage of the digestion, but which still interact with subtilopeptidase A, *e.g.*, RNase S and 41-DNP RNase S, will require an examination of the dependence of the initial velocities of the digestion upon the concentrations of the two proteins.

Crystallization of 41-DNP RNase S. Crystals of 41-DNP RNase S of a habit and order suitable for high-resolution X-ray diffraction studies were readily grown at pH 5.5 from 40% saturated ammonium sulfate solutions which also contained 3 M cesium chloride. The space group of these crystals is the same as that of crystals of RNase S grown under identical conditions, *viz.*, $P3_121$. Moreover, the unit cell dimensions

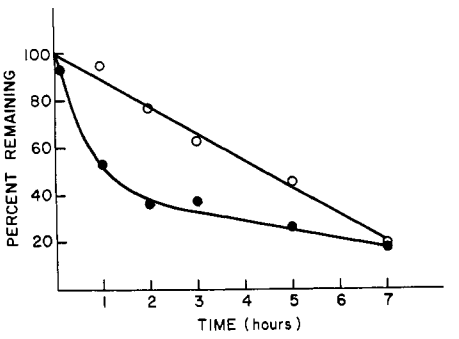

FIGURE 6: Time course of the subtilopeptidase A digestion of RNase A(○) and of 41-DNP RNase A (●). See Methods for experimental details.

TABLE III: Unit Cell Dimensions of Crystals of RNase S and of 41-DNP RNase S. The Space Group in Both Cases Is $P3_121$ (Trigonal).

	a (*b*) (Å)	*c* (Å)
RNase S	44.5	97.3
41-DNP RNase S	44.9	97.6

are nearly identical with those of the RNase S crystals (Table III).

Attempts to crystallize 41-DNP RNase A under a variety of conditions were uniformly unsuccessful.

Structure in the Crystal of 41-DNP RNase S. The structure in the crystal of 41-DNP RNase S has been determined to 3-Å resolution; a comparison of this structure with that of RNase S (Wyckoff *et al.*, 1970) has revealed that the "positon of the DNP group does not block the active site in any way, although it clearly requires a shift of the side chains of Lys-41 and Arg-39" (Allewell, 1969). In addition, numerous smaller shifts in positioning occur at widely separated locations in the molecule. Some of these alterations probably represent changes in the interactions between neighboring molecules as a result of the presence of the DNP group; whether all these latter changes can be explained by this mechanism is uncertain at this time. A striking feature noted by Allewell was the diminution of many of the structural differences between native RNase S and the derivative upon the diffusion into the crystal of the inhibitor cytidine 3′-monophosphate. A detailed discussion of the nature of the structural differences between RNase S and 41-DNP RNase S will be the subject of a separate publication (N. Allewell and H. W. Wyckoff manuscript in preparation).

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