On the Structure of 41-Dinitrophenyl Ribonuclease A. Solvent Perturbation, Thermal Transition, Optical Rotatory Dispersion, and Binding Studies*

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ABSTRACT: To further describe the inactivation of ribonuclease A by dinitrophenylation at lysine-41, solvent perturbation, thermal transition, optical rotatory dispersion, and binding properties were studied. Utilizing the hydantoin of ε-dinitrophenyllysine as a model for a completely accessible dinitrophenyl moiety, solvent perturbation with 20% ethylene glycol revealed that the dinitrophenyl group in 41-dinitrophenyl ribonuclease A is at least paritally folded into the protein matrix. Thermal transition measurements provided substantiation, since a dinitrophenyl difference spectrum appeared on heat denaturation of the protein. Optical rotatory dispersion and thermal transition measurements also revealed possible small conformational differences between ribonuclease A and 41-dinitrophenyl ribonuclease A. 41-Dinitrophenyl ribonuclease A binds cytidine 3′-monophosphate but the binding affinity was found to be significantly less than with ribonuclease A at pH 5.5. An interpretation of the results is offered in which the lack of activity of 41-dinitrophenyl ribonuclease A is ascribed to diminished binding affinity and impaired catalytic efficiency that result from the lowered basicity of the anion-binding site and from small conformation changes.

Studies of the dinitrophenylation of bovine pancreatic ribonuclease A have suggested that changes in conformation and anion-binding capacity occur concomitant with inactivation by reaction at lysine-41 (Hirs *et al.*, 1965; Murdock *et al.*, 1966). Conformational alterations were inferred from the dependence of reaction at lysine-7 upon prior reaction at lysine-41. Diminished anion-binding capacity appeared to be a plausible explanation for the chromatographic behavior on IRC-50 of 41-DNP-RNase A relative to that of 1-α-DNP-RNase A. That lysine-41 is at or near the anion-binding site was inferred from the observation that phosphate and other competitive inhibitors of RNase (Ukita *et al.*, 1961) markedly reduce the rate of inactivation.

The present experiments were performed to attain a more precise description of the inactivation process and associated changes. New information about the conformation and binding capacity of 41-DNP-RNase A relative to that of unmodified RNase A was obtained by thermal transition, optical rotatory dispersion, and equilibrium binding studies. In addition the environment of the DNP moiety in 41-DNP-RNase A was probed by the solvent perturbation method.

Materials and Methods

Bovine RNase A (Worthington Biochemical Corp.), in phosphate buffer with phenol preservative, was further fractionated by chromatography over sulfoethyl-Sephadex C-25 (medium; Pharmacia) in 0.1 M sodium phosphate buffer at pH 6.30 to remove contaminants eluted before the principal protein fraction (Fruchter and Crestfield, 1965). A mixture of 2′- and 3′-CMP (Schwarz Bioresearch, Inc.) was separated by chromatography on Dowex 1-X10 by a modification of the procedure of Cohn (1950). The chloride form of the resin was converted into the formate form by successive equilibrations with saturated sodium acetate solution, 0.05 M formic acid, and finally deionized water. Quantities of 170–190 mg of the mixed isomers in 30 ml of 0.005 M formic acid were absorbed on a column (2.5 × 100 cm) which was washed with deionized water until the conductivity of the eluate remained constant. Subsequent separation of the 2′- and 3′-CMP was accomplished with 0.05 M formic acid as eluent. Paper chromatography of the 3′-CMP in saturated ammonium sulfate–1 M sodium acetate–isopropyl alcohol (80:18:2) (Markham and Smith, 1952) established the absence of 2′-CMP. Reagent grade dioxane was redistilled over sodium.

The following extinction coefficients were assumed for spectrophotometric purposes: RNase A, $E^{1\%}_{1\,cm}$ 6.95 at 280 mμ (Sherwood and Potts, 1965); 3′-CMP, ε 9400 at 271 mμ, pH 7 (Harris *et al.*, 1953); 41-DNP-RNase A, $E^{1\%}_{1\,cm}$ 11.2 at 280 mμ determined by the method of Lowry (Lowry *et al.*, 1951) with RNase A as standard.

Previously described procedures were utilized for the reaction of fluorodinitrobenzene with RNase A and subsequent isolation of 41-DNP-RNase A (Hirs *et al.*, 1965). Preparations of 41-DNP-RNase A used had approximately 1% the activity of native RNase when as-

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sayed with cyclic 2',3'-CMP (Schwarz Bioresearch Corp.) (Murdock *et al.*, 1966).

Solvent perturbation measurements (Herskovits and Laskowski, 1962) were made with a Cary 14 spectrophotometer. The cell compartment was maintained at 25.0°; scans were made from 600 to 250 mμ. The trimming potentiometers were adjusted each day with the appropriate cells, buffers, and solvents to obtain a reliable base line. Ethylene glycol (Matheson Coleman and Bell) (20%) was used as the perturbant; all experiments were performed in 0.1 M sodium acetate buffer (pH 5.5). Specially constructed cell holders permitted exact reproducibility in positioning double-compartment cylindrical cells of the type described by Herskovits and Laskowski. As a model for perturbation experiments with the DNP moiety, the hydantoin of ε-DNP-lysine was synthesized by the method of Stark and Smyth (1963). Its structure was confirmed by infrared and elemental analyses. Concentrations of the hydantoin of DNP-lysine and of 41-DNP-RNase A were 1.3×10^{-4} M; the absorbance at 280 mμ was approximately 2. Spectra are reported in terms of the negative value of observed absorption differences.

For the thermal transition studies reverse difference spectra were recorded from a Cary 14 spectrophotometer by heating a jacketed rectangular cell in the reference compartment from 25 to 80° while maintaining the cell in the sample compartment at 25°. One pair of solutions was used for the measurement of an entire thermal transition. At each temperature, difference spectra were recorded from 330 to 260 mμ with RNase A and from 600 to 260 mμ with 41-DNP-RNase A. Solutions were used with 1×10^{-4} M RNase A or 7×10^{-5} M 41-DNP-RNase A at an absorbancy of 1.00 at 280 mμ in 0.009 M Tris-HCl buffer at pH 7.2. The pH dependence of the temperature transition is small at neutrality (Hermans and Scheraga, 1961). Small errors in T_M determinations by this method which arise from irreversibility in the thermal transition (von Hippel and Wong, 1965) did not affect the interpretations.

Optical rotatory dispersion spectra were obtained with a Cary 60 spectropolarimeter in the range from 600 to 190 mμ. In regions where Cotton effects were noted or predicted overlapping recordings were obtained with cells having different path lengths and/or by altering the range of the rotation scale. A 1-cm cell was used from 600 to 260 mμ, a 0.1 -cm cell from 260 to 215 mμ, and a 0.01-cm cell from 240 to 190 mμ. Concentrations of RNase A and 41-DNP-RNase A, 1.6 and 1.11×10^{-4} M, respectively, in 0.1 M sodium acetate buffer (pH 5.5) were utilized to obtain suitable spectra at these path lengths. Spectra were recorded in terms of molar rotations without refractive index corrections.

The binding of 3'-CMP to RNase A or 41-DNP-RNase A was studied by a modification of the difference spectrum procedure described by Hummel *et al.* (1961). Reverse difference spectra were recorded in a Cary 14 spectrophotometer equipped with the double-compartment cylindrical cells used in the solvent perturbation work. In the reference compartment the cell contained buffer in one chamber, 3'-CMP and RNase A or 41-DNP-RNase A in the other. The sample compartment cell contained 3'-CMP and RNase A or 41-DNP-RNase A in separate chambers. Experiments were performed at 25.0° in 0.1 M sodium acetate buffer (pH 5.5), conditions reported as optimal for binding (Hammes and Schimmel, 1965). Concentrations of 3'-CMP were varied from 1×10^{-5} to 1.2×10^{-4} M with RNase A and from 3×10^{-5} to 2.0×10^{-4} M with 41-DNP-RNase A. Consecutive additions of 3'-CMP with 10- or 20-μl pipets were made to 2.8 or 4.7×10^{-5} M protein solution to obtain the desired 3'-CMP concentrations. Corrections were made for dilution. The solutions were mixed magnetically and the 1/8-in. bars were left in the cells, where they did not interfere with the light beams. The base line was adjusted in the presence of the stirring bars. These binding measurements were complemented with semiquantitative measurements of the binding capacity of 41-DNP-RNase A and RNase A by a Sephadex dialysis technique (Fasella *et al.*, 1965).

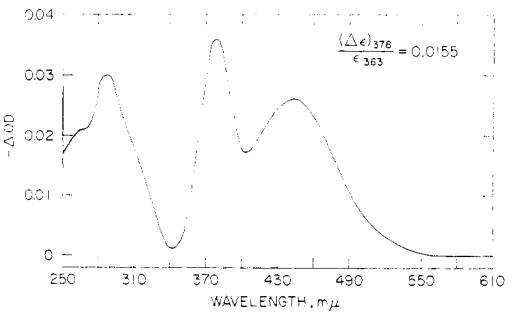

FIGURE 1: The solvent perturbation difference spectrum of the hydantoin of ε-DNP-lysine (1.35×10^{-4} M) in 20% ethylene glycol–0.1 M sodium acetate buffer (pH 5.5). The negative of the observed absorbance difference, $-\Delta OD$, is plotted against wavelength. $(\Delta\epsilon)_{378}$ is the molar difference extinction coefficient at 378 mμ, ϵ_{363} is the molar extinction coefficient at 363 mμ. The value of $(\Delta\epsilon)_{378}/\epsilon_{363}$ is an average value from duplicate difference spectra on five separate occasions.

Results

The presence of the DNP group in 41-DNP-RNase gives rise to a principal absorption peak at 363 mμ with a shoulder at higher wavelengths. The absorption spectrum of the hydantoin of DNP-lysine also shows a similar peak at 363 mμ. Solvent perturbation of the hydantoin of DNP-lysine in 20% ethylene glycol gives rise to a difference spectrum (Figure 1) indicative of a blue shift in the absorption of the DNP chromophore, opposite to the shift normally observed with tyrosine residues in proteins (Herskovits and Laskowski, 1962). This is evidenced by the negative difference peak at 378 mμ. Difference peaks are observed at the identical wavelength on perturbation of 41-DNP-RNase A in 20% ethylene glycol (Figure 2). The normally observed tyrosine perturbation peaks appear at 287–288 and 268–270 mμ. The appreciable overlap from the DNP difference peaks prevented quantitative comparison of the tyrosine difference peaks to those of RNase A itself. A convenient reduced parameter obtained from solvent perturbation difference spectra is the ratio of the absorb-

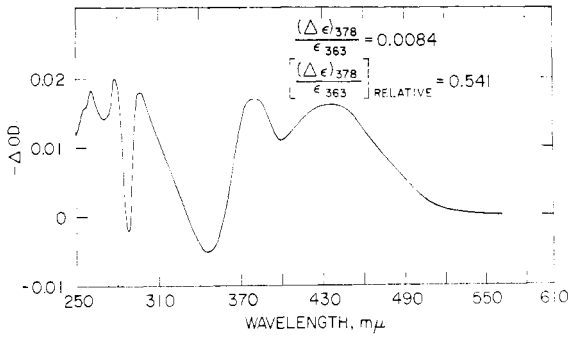

FIGURE 2: The solvent perturbation difference spectrum of 41-DNP-RNase A (1.38×10^{-4} M) in 20% ethylene glycol–0.1 M sodium acetate buffer (pH 5.5). The negative of the observed absorbance difference is plotted against wavelength. $(\Delta\epsilon)_{378}$ is the molar difference extinction coefficient at 378 mμ, ϵ_{363} the molar extinction coefficient at 363 mμ, and $(\Delta\epsilon)_{378}/\epsilon_{363}$ relative is the ratio of $(\Delta\epsilon)_{378}/\epsilon_{363}$ for 41-DNP-RNase A and DNP-lysinehydantoin. The value of $(\Delta\epsilon)_{378}/\epsilon_{363}$ is an average value from duplicate difference spectra on two separate occasions.

ance at a difference peak to the absorbance of the same concentration of chromophore in the absence of perturbant (Herskovitz and Laskowski, 1962). This of course is equal to the ratio of the molar difference extinction coefficient to the molar absorptivity, $\Delta\epsilon/\epsilon$. For the difference peak at 378 mμ and the absorption peak at 363 mμ the ratio is 0.0155 for the hydantoin of DNP-lysine and 0.0084 for 41-DNP-RNase A. Since difference peaks were observed at identical wavelengths with 41-DNP-RNase A and the hydantoin, the relative value of the ratios, $(\Delta\epsilon)_{378}/\epsilon_{363}$ relative, should provide a valid estimate of the relative exposure of the DNP-moiety in 41-DNP-RNase A. The value obtained, 0.54, reveals that the DNP group in 41-DNP-RNase A is at least partially buried within the protein matrix.

Further substantiation came from thermal transition spectra. The difference spectrum between 41-DNP-RNase A at 25 and 59.4° (Figure 3) shows a conspicuous negative peak at 358 mμ due to exposure of the DNP group when the protein unfolds. The existence of a difference spectrum in the region around 360 mμ is further evidence that the DNP chromophore is at least partially enclosed within the protein structure in its native state. Again, in contrast to the shifts commonly noted with tyrosine in proteins, a red shift occurs with exposure of the DNP moiety to a more aqueous environment. Tyrosine difference peaks at 279.5 and 286.5 mμ, as observed in RNase A, are also observed in the difference spectrum that arises from heat denaturation of 41-DNP-RNase A. Difference peaks at lower wavelengths apparently due to phenylalanine were also evident in the present measurements of the difference spectrum for RNase A. To compare the heat stabilities of 41-DNP-RNase A and the parent protein, the negative difference in absorbance at a series of temperatures relative to the maximal difference absorbance obtained at 286.5 mμ was plotted against temperature (Figure 4). The T_M or midpoint of the thermal transition measured in this manner is 62.1° for RNase A and 61.9° for 41-DNP-RNase A, an insignificant difference. Thus, it appears that no gross conformation changes ensue from dinitrophenylation of RNase A at lysine-41. It is of considerable interest (Figure 4) that when the transition for the exposure of the DNP moiety is similarly traced, from the difference peak at 358 mμ, the transition is complex and "T_M" is 55.5°, indicative of a relatively higher exposure at lower temperatures of the DNP group than of buried tyrosines. This fact, coupled with the observation that maximal exposure occurs only in congruence with maximal tyrosine exposure, suggests that in the thermal transition the 41-DNP-RNase A molecule unfolds in stages. This observation does not support a two-state hypothesis for the temperature denaturation of

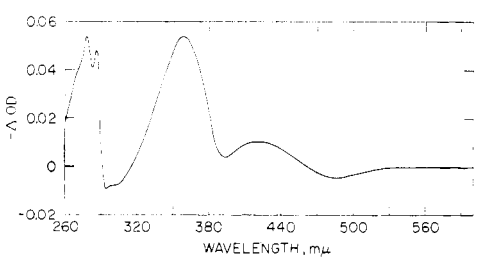

FIGURE 3: The reverse difference spectrum from 41-DNP-RNase A (7.42×10^{-5} M) at 25.0 and 59.4° in 0.009 M Tris-HCl buffer (pH 7.2). Solutions were heated in jacketed cells and the spectra were recorded directly.

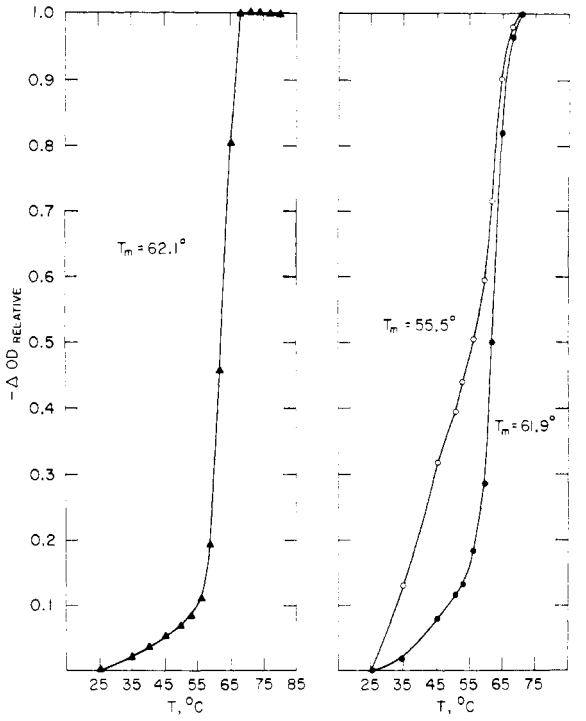

FIGURE 4: Thermal transition curves for RNase A (▲—▲) and 41-DNP-RNase A (●—●) from the difference absorbance at 286.5 mμ, and for 41-DNP-RNase A (○—○) from the difference absorbance at 358 mμ. $-\Delta OD_{rel}$ is the ratio of the negative difference absorbance at each temperature to the maximal difference absorbance obtained by heat denaturation. T_M is an average value from two experiments with each protein of the midpoint of the transition. The measurements were conducted in 0.009 M Tris-HCl buffer (pH 7.2). Concentrations: RNase A, 1.11×10^{-4} M; 41-DNP-RNase A, 7.22×10^{-5} M.

RNase A (Brandts and Hunt, 1967; Lumry *et al.*, 1966) and is in agreement with other observations inconsistent with this hypothesis (Klee, 1967; Poland and Scheraga, 1965).

Optical rotatory dispersion measurements were undertaken for the twofold purpose of searching for possible Cotton effects associated with the DNP group and for a general conformational comparison of RNase A and 41-DNP-RNase A. When the optical rotatory dispersion spectra from 600 to 300 mμ were compared (Figure 5), anomalous dispersion was present in the region from 350 to 450 mμ in the optical rotatory dispersion spectrum for 41-DNP-RNase A. This arises from at least one positive Cotton effect associated with the DNP group. Small differences in the optical rotatory dispersion spectra were noted throughout the spectral region from 600 to 190 mμ (Figures 5 and 6) including an approximate 8% difference at the 228-mμ trough. The magnitude of the differences is a further indication that no gross conformational changes occur on dinitrophenylation at lysine-41. It is likely that the observed small differences demonstrate actual conformational differences between the proteins in addition to contributions throughout the 41-DNP-RNase A spectrum from Cotton effects due to the DNP group.

Germane to an investigation of the manner in which the enzyme is inactivated by dinitrophenylation are measurements of substrate-binding capacity. For this purpose use was made of the difference spectrum observed when 3′-CMP binds to RNase A. This spectrum exhibits a negative difference peak at 264 mμ (Hummel *et al.*, 1961; Mathias *et al.*, 1960). It has been suggested that this difference spectrum results primarily from a perturbation of the cytidine moiety (Hummel *et al.*, 1961). Some additional support for this contention was derived from the observation that the difference spectrum generated by solvent perturbation of 3′-CMP in 80% dioxane–water is similar to that obtained when 3′-CMP binds to RNase A. Addition of 3′-CMP to 41-DNP-RNase A also generates a difference spectrum with a negative maximum at 264 mμ (Figure 7). This shows that 41-DNP-RNase A in fact binds 3′-CMP. Since qualitative differences are evident around 280 mμ in the respective difference spectra, it is unlikely that the observed difference spectrum with 41-DNP-RNase A is due to contaminating unmodified RNase A in the sample of 41-DNP-RNase A studied. The magnitude of the difference absorbance at 264 mμ was measured as a function of the concentration of 3′-CMP at constant concentration of RNase A or 41-DNP-RNase A. The results were analyzed according to

$$K_{diss} = \frac{\left(C_0 - \frac{\Delta OD_{264}}{(\Delta\epsilon)_{264}}\right)\left(R_0 - \frac{\Delta OD_{264}}{(\Delta\epsilon)_{264}}\right)}{\frac{\Delta OD_{264}}{(\Delta\epsilon)_{264}}}$$

where K_{diss} is the dissociation constant for the complex of 3′-CMP with either RNase A or 41-DNP-RNase A, ΔOD_{264} is the observed difference absorbance at 264 mμ at a particular concentration of complex, $(\Delta\epsilon)_{264}$ is the molar difference absorptivity at 264 mμ of the 3′-CMP–protein complex, and C_0, R_0 are the initial concentrations

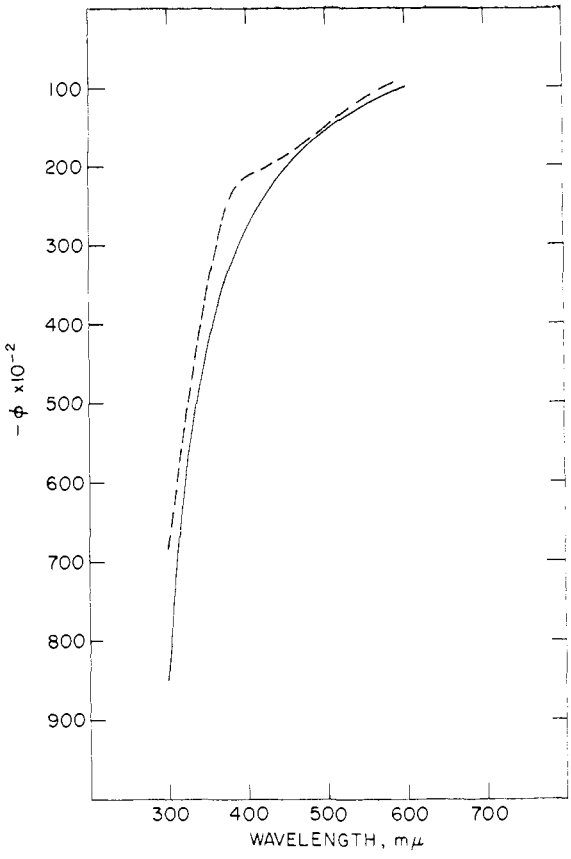

FIGURE 5: The optical rotatory dispersion spectra of RNase A (——) and 41-DNP-RNase A (– – –) in 0.1 M sodium acetate buffer (pH 5.5). ϕ is the molar rotation in deg cm^{-1} M^{-1}. Concentrations: RNase A, 1.6×10^{-4} M; 41-DNP-RNase A, 1.11×10^{-4} M. In regions where Cotton effects were noted or predicted overlapping recordings were obtained with cells having difference path lengths and/or by altering the range of the rotation scale.

of 3′-CMP and RNase A or 41-DNP-RNase A, respectively. A nonlinear least-squares program[1] with minimization of the difference between observed and calculated ΔOD was used to solve directly for the values of K_{diss} and $(\Delta\epsilon)_{264}$. Since, as will be demonstrated, there is perturbation of the spectrum of the DNP group in the 3′-CMP–41-DNP-RNase A complex, the values of $(\Delta\epsilon)_{264}$ for RNase A and 41-DNP-RNase A must be determined independently. The results (Table I) indicate that although 3′-CMP binds to 41-DNP RNase A, the dissociation constant is considerably higher than that observed for the interaction of 3′-CMP with RNase A itself. Semiquantitative confirmation for reduced binding affinity was obtained from Sephadex dialysis experiments at pH 5.5 in which binding of 3′-CMP to 41-DNP-RNase A could not be distinguished significantly from zero binding.

It is noteworthy that the difference spectra of the 3′-CMP–41-DNP-RNase A complex revealed that perturbation of the spectrum of the DNP group occurs (Figure 8). This difference spectrum due to the DNP group resembles that observed in the thermal transition

[1] IBM share program no. SDA 3094 by D. W. Marquardt.

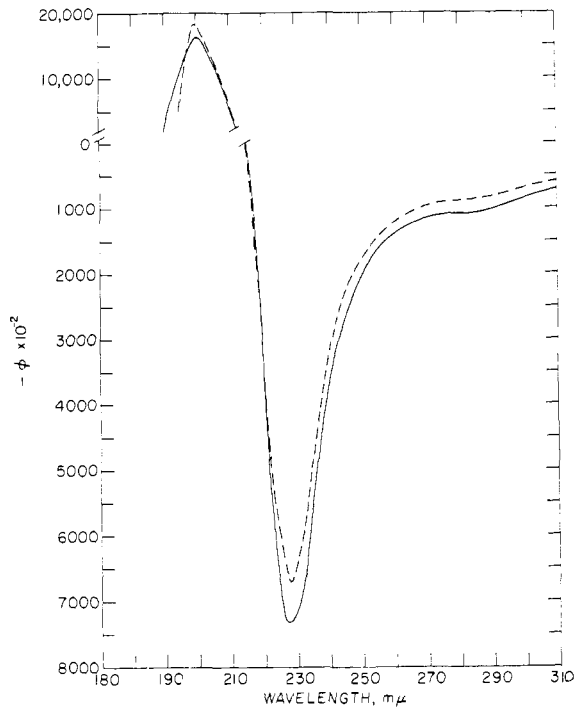

FIGURE 6: The optical rotatory dispersion spectra of RNase A (——) and 41-DNP-RNase A (– – –) in 0.1 M sodium acetate buffer (pH 5.5). ϕ is the molar rotation in deg cm^{-1} M^{-1}. Concentrations: RNase A, 1.6×10^{-4} M; 41-DNP-RNase A, 1.11×10^{-4} M. In regions where Cotton effects were noted or predicted overlapping recordings were obtained with cells having different path lengths and/or by altering the range of the rotation scale.

of 41-DNP-RNase A (Figure 3) and suggests that the DNP group becomes more exposed to solvent concomitant with 3′-CMP binding to 41-DNP-RNase A.

Discussion

The rate of inactivation when RNase A is dinitrophenylated is virtually identical with the rate at which lysine-41 is modified (Murdock *et al.*, 1966); known competitive inhibitors of RNase markedly reduce the rate of inactivation (Hirs *et al.*, 1965). Lysine-41 is therefore most likely located at or near the active site, a view substantiated by the results of recent X-ray work with RNase A and S (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967). Inactivation by reaction at lysine-41 must occur as a consequence of alterations in either binding, catalysis, or both. Possible contributions to altered binding and catalysis include steric hindrance by the DNP moiety, marked reduction in basicity of the amino group of lysine-41, and conformational changes. The experiments reported dwell on these subjects.

The value for the dissociation constant at pH 5.5 and 25° for the complex formed by RNase A and 3′-CMP in the present experiments is in fair agreement with values obtained previously by other workers (Hammes and Schimmel, 1965; Herries *et al.*, 1962; Deavin *et al.*, 1966). They showed that maximum binding occurs at pH 5.5. That 41-DNP-RNase A and 3′-CMP interact at this pH was shown qualitatively by the appearance of a difference spectrum which revealed the presence of spectral perturbations in both the nucleotide and DNP chromophores. While the values obtained by measurement of $-(\Delta\epsilon)_{264}$ for RNase A and 41-DNP-RNase A were subject to unavoidable errors (*cf.* Table I), the dissociation constant for the complex of 41-DNP-RNase A with 3′-CMP is clearly between one and two orders of magnitude larger than for the corresponding complex with RNase A itself. This finding supports previous suggestions (Hirs *et al.*, 1965) that dinitrophenylation of lysine-41 diminishes anion binding and that lysine-41 is an integral part of the anion-binding site in RNase.

TABLE I: Interaction of 3′-CMP with RNase A and 41-DNP-RNase A.[a]

	$K_{diss} \times 10^4$ (M)	$-(\Delta\epsilon)$ 264 mμ (M^{-1} cm^{-1})
RNase A	0.12 ± 0.05	2648 ± 208
41-DNP-RNase A	2.2 ± 0.8	1926 ± 454

[a] The solvent was 0.1 M sodium acetate buffer (pH 5.5) at 25°. Ten concentrations of 3′-CMP from 1×10^{-5} to 1.2×10^{-4} M were used with RNase A (2.8 or 4.7×10^{-5} M) on three separate occasions and five concentrations from 3×10^{-5} to 2×10^{-4} M with 41-DNP-RNase A (4.7×10^{-5} M). At each concentration of 3′-CMP $(\Delta OD)_{264\,m\mu}$ for the calculations was the average value obtained from at least two scans of the difference spectrum. A nonlinear least-squares program was used to obtain the dissociation constants for the interactions, K_{diss}, and the molar difference extinction coefficient, $\Delta\epsilon$, at 264 mμ with equal weight given to ΔOD values obtained at each 3′-CMP concentration. The standard errors for the determinations are also indicated.

The difference in affinity between RNase A and 41-DNP-RNase A for 3′-CMP and cyclic CMP would probably be less pronounced under the conditions prevalent in the usual kinetic assays for the enzyme at pH 7 (Herries *et al.*, 1962). Furthermore, inasmuch as 41-DNP-RNase A preparations have been obtained with specific activities no greater than 0.2% of the specific activity of the parent protein, it is evident that the activity of 41-DNP-RNase A is substantially smaller than would be expected from the reduction in binding affinity alone. Indeed, since the activity of 41-DNP-RNase A varies with the extent of fractionation, the intrinsic activity of the protein in fact may be significantly less than has hitherto been observed. Thus, the conclusion is inescapable that dinitrophenylation of RNase A at lysine-41 inactivates by a combination of two processes: by reduction of the binding affinity for the substrate and by impairment of catalytic efficiency.

It is possible that the diminished basicity which must result from substitution of the amino group of lysine-41 would be sufficient to account quantitatively for the observed effects on binding and catalysis. This account

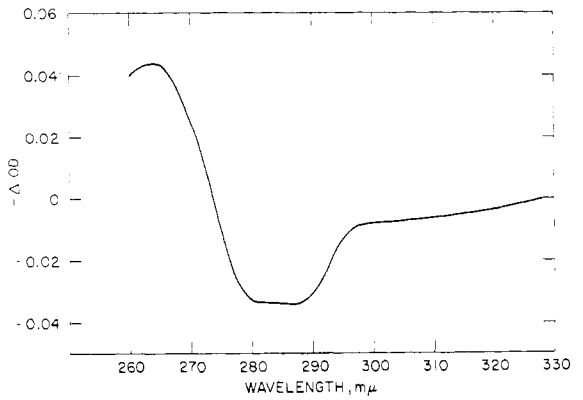

FIGURE 7: Reverse difference spectrum for the binding of 3′-CMP (1.21×10^{-4} M) to 41-DNP-RNase A (4.59×10^{-5} M) in 0.1 M sodium acetate buffer (pH 5.5).

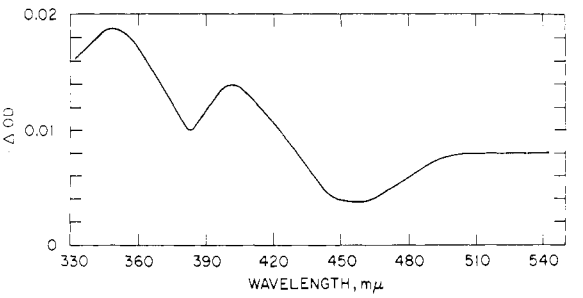

FIGURE 8: Reverse difference spectrum on binding of 3′-CMP (1.09×10^{-4} M) to 41-DNP-RNase A (4.13×10^{-5} M) in 0.1 M sodium acetate buffer (pH 5.5).

would visualize the positively charged ε-amino group of lysine-41 participating directly in both binding and catalysis. However, the solvent perturbation and thermal transition measurements show that the DNP group in 41-DNP-RNase A is partially folded into the protein matrix. The observations correlate with those of Brown *et al.* (1967), who found that anti-DNP antibody reacts only poorly with 41-DNP-RNase A. Moreover, the dependence of dinitrophenylation at lysine-7 on prior reaction at lysine-41 (Hirs *et al.*, 1965) was previously interpreted in terms of a structural rearrangement. Accommodation of the DNP group at position 41 in the surface configuration of the protein would require a local rearrangement of the structure and would serve to immobilize the side chain of lysine-41. The attainment of the catalytic efficiency that is so characteristic of enzymes requires that adsorbed substrate molecules be aligned precisely relative to those functional groups that participate in the development of the transition state (Koshland, 1962). Even relatively small distortions of the active site would be sufficient to affect efficiency dramatically. Such considerations require that the distortions introduced in the protein around lysine-41 must also be accepted as potentially significant contributors to the inactivation process. The optical rotatory dispersion and thermal transition data, in excellent agreement with the results reported by others for RNase A itself (Cathou *et al.*, 1965; Harrington and Schellman, 1956; Hermans and Scheraga, 1961; von Hippel and Wong, 1965), serve to confirm that no gross conformation changes accompany introduction of a DNP group at lysine-41. However, the small differences between 41-DNP-RNase A and the parent protein that were actually observed are consistent with the localized changes contemplated in the present discussion.

It is unlikely that a relatively limited conformational change would have an important influence on the strength of substrate binding, but such a change could affect catalytic efficiency drastically. In this context it is worth recalling that purine and pyrimidine nucleotides are strongly bound to RNase A (Ukita *et al.*, 1961) as are to a lesser degree the corresponding nucleosides, but only pyrimidine nucleotides serve as substrates. It may be presumed that when purine nucleotides are bound catalysis fails to ensue because the correct apposition of functional groups on the enzyme and on bound substrate molecules cannot be achieved. An analogous situation may arise when substrates are bound to 41-DNP-RNase A, except that in this instance the critical alignments between groups on the enzyme and the substrate may no longer be possible because the active site has been distorted.

A discussion of the structure of 41-DNP-RNase A must take cognizance of the relative size of the DNP group, larger than a pyrimidine base, approximately half the volume of a pyrimidine nucleoside cyclic phosphate substrate. Incorporation of so bulky a group into the active site of RNase would constitute a functionally unacceptable perturbation in that it would preclude any possibility of binding. From this standpoint it is more acceptable to postulate that the DNP group is incorporated into the structure in a region somewhat peripheral to the active site and that the distortion of the site itself arises as a result of a more general rearrangement of the molecule in the region of lysine-41. This view is favored by the nature of the difference spectrum that arises when 41-DNP-RNase A complexes with 3′-CMP. This spectrum shows that the DNP group behaves in the same manner on complexing as it does when the protein undergoes its thermal transition; *i.e.*, the behavior is consistent with a greater exposure of the chromophore to the solvent environment. Were the DNP group completely incorporated into the active site, it would be unlikely that binding of 3′-CMP would afford an opportunity for its exposure. That binding of 3′-CMP induces an exposure of the DNP moiety is also further evidence for a protein conformation change on 3′-CMP binding to RNase A (Cathou and Hammes, 1964; Cathou *et al.*, 1965).

Finally, it is noteworthy that the results and interpretations presented in this paper are consistent with the fundamental structural information made available by X-ray crystallography (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967). 41-DNP-RNase S is essentially, but not completely, isomorphous with RNase S (H. W. Wyckoff, personal communication) and it has been possible to obtain a difference Fourier map from which it is evident that the DNP group is folded into the structure in a region removed from the active site. The difference Fourier reveals the presence of a large number of difference peaks and shows that (at the resolution attained)

incorporation of the DNP group introduces relatively small distortions throughout the whole molecule. It is noteworthy, moreover, that when crystals of 41-DNP-RNase S are soaked with nucleotides, binding is seen to occur in the same site as in RNase S.

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