Biol. 53, 401.
Riggs, A. D., Bourgeois, S., Newby, R. F., and Cohn, M. (1968), *J. Mol. Biol. 34,* 365.
Riggs, A. D., Suzuki, H., and Bourgeois, S. (1970a), *J. Mol. Biol. 48,* 67.
Sevall, J. S., Cockburn, A., Savage, M., and Bonner, J. (1975), *Biochemistry 14,* 782.
Sober, H. A., Ed., (1968), Handbook of Biochemistry, Cleveland, Ohio, Chemical Rubber Co.
Stein, G., Stein, J., Thrall, C., and Park, W. (1975), in Chromosomal Proteins and Their Role in the Regulation of Gene Expression, Stein, G. S., and Kleinsmith, L. J., Ed., New York, N.Y., Academic Press, p 1.
Studier, W. F. (1965), *J. Mol. Biol. 11,* 373.
Teng, C. S., Teng, C. T., and Allfrey, V. G. (1971), *J. Biol. Chem. 246,* 3597.
Thomas, T. L., Allen, M. A., and Patel, G. L. (1973), *J. Cell Biol. 59,* 345a.
Thomas, T. L., and Patel, G. L. (1974), *J. Cell Biol. 63,* 347a.
van den Broeck, H. W. J., Nooden, L. D., Sevall, J. S., and Bonner, J. (1973), *Biochemistry 12,* 229.
von Hippel, P. H., and McGhee, J. D. (1972), *Annu. Rev. Biochem. 41,* 231.
Wang, J. C., Barkley, M. D., and Bourgeois, S. (1974), *Nature (London) 251,* 247.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310,* 384.
Yamamoto, K. R., and Alberts, B. M. (1970), *Virology 40,* 734.

Photochemical Cross-Linking of Neighboring Residues in Protein–Nucleic Acid Complexes: RNase and Pyrimidine Nucleotide Inhibitors†

Joseph Sperling* and Abraham Havron

ABSTRACT: Irradiation of the stable complexes formed between RNase and its competitive inhibitors cytidine 2′(3′),5′-diphosphate (pCp), and uridine 2′(3′),5′-diphosphate (pUp), resulted in covalent bond formation between the pyrimidine nucleotides and the enzyme. The photochemical reactions were initiated by ultraviolet light of $\lambda > 300$ nm, employing acetone as a photosensitizer. This method was found to yield less undesired by-products, particularly photolyzed amino acids and aggregates resulting from protein-to-protein cross-linking, than the direct method of irradiation with light of $\lambda > 260$ nm. Tryptic digestion of the modified protein, and chromatographic analysis of the peptides thus obtained, revealed a single and specific peptide which became covalently linked to both nucleotide inhibitors. The amino acid composition of this peptide is consistent with the sequence Asn-67-Arg-85 of RNase. Part of this peptide (residues 78 through 83) is close to the enzyme's binding site for the pyrimidine moiety of the nucleotides. Denatured RNase failed to cross-link to the inhibitors, and the extent of pUp cross-linking could be reduced by the addition of another competitive inhibitor (3′-UMP). Finally, the presence of excess inhibitor in the irradiation mixture did not lead to nonspecific cross-linking. This indicates that specificity is also achieved due to the fact that unbound excited inhibitor molecules do not react with the protein but prefer to follow different and faster reaction paths.

The active study of protein–nucleic acid interactions in recent years has emphasized the critical role these interactions play in life processes. The control and regulation of gene expression in cellular metabolism involve, for example, protein–DNA interactions (von Hippel and McGhee, 1972), while the protein synthesis machinery involves a variety of protein–RNA interactions (Kurland, 1972). In order to account for the high affinities observed in protein–nucleic acid complexes, simultaneous interactions of a number of functional groups on the two partners must be involved. These groups must be positioned in a specific conformational relationship which permits a favorable interaction to take place. The molecular mechanism upon which these highly developed specific interactions are based is not understood in detail for any of these systems.

Photochemistry provides a promising approach to the study of such interactions since proteins and nucleic acids cross-link covalently when irradiated with ultraviolet light. This has been shown in several systems including DNA and proteins in bacteria (Smith, 1975), bovine serum albumin and DNA (Smith, 1964; Braun and Merrick, 1975), DNA polymerase and DNA (Markovitz, 1972), RNA polymerase and Br-substituted DNA (Weintraub, 1973), the *lac* repressor and the *lac* operon-containing Br-substituted DNA (Lin and Riggs, 1974), and a variety of complexes of amino acyl tRNA synthetases and their cognate tRNAs (Schoemaker and Schimmel, 1974; Budzik et al., 1975; Schoemaker et al., 1975). The proposed approach to the problem of protein–nucleic acid interactions utilizes photochemistry in an attempt to "freeze" existing contact points in protein–nucleic acid complexes, and thereby allowing the identification and chemical characterization of the interacting residues. The major advantage of this approach is that photo-

† From the Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, Israel. A preliminary report was presented at the annual meeting of the Israel Biochemical Society, 1975. *Received September 18, 1975.*

chemical cross-linking can be performed on naturally occurring protein–nucleic acid complexes, under optimal conditions where maximum binding or stability of the native complex occurs, without any need for decomposition, chemical modification, and subsequent reconstitution steps. Further, in irradiation-induced cross-linking, covalent bonds can, in principle, be formed between a large variety of amino acids and either pyrimidines (Smith and Meun, 1968; Gorelic et al., 1972; Jellinek and Johns, 1970; Varghese, 1974) or purines (Ben-Ishai et al., 1973; Sperling, 1976). This point is of major importance, particularly when interactions between long stretches of both amino acid sequences and nucleotide sequences are involved in the protein–nucleic acid complexes.

Certain caution must, however, be taken before utilizing photo-induced cross-linking as a general tool in the study of protein–nucleic acid interactions. The proposed approach is based on the assumption that the covalent bond is formed between an amino acid residue and a base residue only when they are in close proximity in the nucleoprotein complex. This means that only excited molecules which are involved in actual binding will become cross-linked, whereas unbound excited molecules will revert to ground state or will follow different and faster reaction paths (e.g., pyrimidine dimer formation).

In order to evaluate this point we have chosen to study, as a test case, the photochemical cross-linking of RNase with pCp[1] and pUp. The structure of RNase has been elucidated by x-ray diffraction (Kartha et al., 1967; Wyckoff et al., 1970), and the binding sites for the substrate related compounds 3′-CMP and 3′-UMP have been mapped on the three dimensional structure of the enzyme (Wyckoff et al., 1970; Richards and Wyckoff, 1971). pCp and pUp are strong competitive inhibitors of RNase ($k_I \sim 10^{-6}$ M: Sawada and Irie, 1969; Gorecki, 1971) and form stable complexes with the enzyme. The study of the photochemical behavior of these systems seems to be appropriate to answer the following questions: (i) is the cross-linking specific and does it involve distinct regions on the enzyme; (ii) do these regions bear any relevance to the known binding sites and structure of the complexes; (iii) and to what extent does nonspecific cross-linking occur?

In this publication we describe experiments in which we have induced covalent bond formation between RNase and its competitive inhibitors pCp and pUp, either by direct ultraviolet irradiation ($\lambda > 260$ nm), or through photosensitization with acetone and light of $\lambda > 300$ nm. We show that a specific covalent cross-link is formed between peptide Asn-67-Arg-85 of RNase and either inhibitor. The same bond is formed exclusively even when a large excess of inhibitor is present, showing that the nonspecific cross-linking is negligible.

Experimental Section

Materials

Bovine pancreatic ribonuclease A (five-times crystallized type 1-A), bovine trypsin (diphenyl carbamoyl chloride treated, type XI), and crystalline cytidine were purchased from Sigma. Uridine was obtained from Aldrich. Uniformly labeled [U-^{14}C]cytidine (451 mCi/mmol, code CFB 55) and [U-^{14}C]uridine (540 mCi/mmol, code CFB 51) were purchased from the Radiochemical Centre, Amersham.

[U-^{14}C]pCp and [U-^{14}C]pUp were prepared by phosphorylating the appropriate U-^{14}C-labeled nucleosides, according to the method described by Hall and Khorana (1955). A modified purification procedure was, however, employed. The reaction mixture of each of the phosphorylated nucleosides was applied to a column of the bicarbonate form of Dowex-2 × 10 and eluted with a linear gradient (0.2–0.8 M) of ammonium bicarbonate solution at pH 8.5. pCp and pUp were eluted at buffer concentrations of 0.52 and 0.55 M, respectively. The pooled fractions from each preparation were lyophilized to yield solid ammonium salts of pCp and pUp in 70–90% yield. These products were pure as shown by thin-layer chromatography on PEI-cellulose sheets (Merck, Darmstadt) developed with 0.2 M ammonium bicarbonate. The R_f value of pCp was 0.55 relative to cytidine, and that of pUp was 0.4 with respect to uridine.

Spectroscopic grade acetone was a product of Fluka, Sephadex G-50 superfine was purchased from Pharmacia, and Dowex-2 × 10 from the Dow Chemical Company. All other chemicals were of analytical grade.

Methods

Amino acid analyses were performed on a Beckman automatic amino acid analyzer, Model 120C, after hydrolysis in 6 N HCl for 22 h at 110 °C in the presence of phenol (1 drop of 5% aqueous solution per hydrolysis tube) to prevent excessive destruction of tyrosine.

Radioactivity measurements were performed on a Packard Model 3003 liquid scintillation spectrometer. Aqueous solutions were counted in Bray's solution (Bray, 1960), whereas chromatographic or electrophoretic papers were thoroughly dried, cut into strips (1.5-cm wide), and counted in toluene scintillation liquid [4 g of 2,5-diphenyloxazole (PPO) and 0.05 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl-POPOP) per 1 l. of freshly distilled toluene].

RNase concentrations were determined spectrophotometrically at 277.5 nm using $\epsilon_{277.5nm}$ 9800 l. mol^{-1} cm^{-1} (Sela and Anfinsen, 1957).

Concentrations of pCp and pUp were determined spectrophotometrically at pH 5.5 using ϵ_{270nm} 9.2 × 10^3 l. mol^{-1} cm^{-1} for pCp, and ϵ_{262nm} 10^4 l. mol^{-1} cm^{-1} for pUp.

Ribonuclease activity was assayed with yeast RNA in 0.1 M acetate buffer at pH 5, according to the method of Anfinsen (1954).

Polyacrylamide gel electrophoreses were carried out with a Shandon apparatus using 10% gels in 0.1 M phosphate buffer, pH 7, containing 0.1% of sodium dodecyl sulfate according to the method of Weber and Osborn (1969).

Trypsin Digestion. Protein samples (5 mg/ml) were dissolved in 0.1 M solution of ammonium bicarbonate at pH 8.5 and digested with trypsin (0.1 mg/ml) for 4 h at 37 °C. The lyophilized digest was dissolved in the appropriate buffer and applied directly to the paper for chromatographic analysis.

High-Voltage Paper Electrophoresis. Material was applied to Whatman No. 3 MM paper and subjected to electrophoresis at 3 kV for 30 min at pH 1.9 or pH 6.5. Longitudinal strips were cut from the paper and developed with ninhydrin–cadmium reagent (Dreyer and Bynum, 1967). Radioactive spots were located as described above.

[1] Abbreviations used are: pUp, uridine 2′(3′),5′-diphosphate; pCp, cytidine 2′(3′),5′-diphosphate; PEI, poly(ethylenimine); PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; 3′-UMP, uridine 3′-monophosphate; 3′-CMP, cytidine 3′-monophosphate.

Paper chromatography was performed on Whatman No. 3MM paper in 1-butanol–acetic acid–water–pyridine (15:3:12:10, v/v) for 20 h.

Irradiations were carried out in a Wild Universal unit (Wild Heerburgg, Switzerland) with a 200-W super pressure mercury lamp (HBO-200 W, Osram, Germany). The samples, in 3-ml spectrophotometric cells, were placed at a distance of 15 cm from the light source in an ice–water cooled jacket and maintained at 25 °C. Heat-absorbing filter consisting of a 2-cm path length quartz cell filled with double-distilled water, was placed in the light path. In photosensitized experiments, a Pyrex cell was used to filter light of $\lambda > 300$ nm.

General Procedure for Cross-Linking of RNase with pUp or pCp. A solution (3 ml) of 0.73 mM RNase, 2.3 mM [U-^{14}C]pUp (specific activity 3.16×10^3 cpm/nmol) or [U-^{14}C]pCp (specific activity 1.4×10^3 cpm/nmol) in 20 mM sodium acetate–acetic acid buffer (pH 5.5) was irradiated for 1 h with light of $\lambda > 260$ nm (Corex filter). In photosensitized experiments, 5% (v/v) acetone was added to the reaction mixture, and light of $\lambda > 300$ nm (Pyrex filter) was employed. The lyophilized reaction mixture was dissolved in 20 mM ammonium bicarbonate (2 ml) and applied to a column (15 × 0.9 cm) of the bicarbonate form of Dowex 2 × 10 equilibrated with the same buffer. Cross-linked RNase and unreacted enzyme were eluted with 20 mM NH_4HCO_3. Unreacted inhibitors and products resulting from dimerization of the pyrimidine nucleotides were subsequently eluted by a linear gradient (0.02–0.8 M) of NH_4HCO_3 (see preparation of pCp and pUp under Materials). The pooled breakthrough fractions were lyophilized, dissolved in 1 ml of 98% formic acid, diluted to 2 ml with water, and subjected to gel filtration (see Figure 4).

A blank reaction consisting of the same mixture of RNase and nucleotide was incubated in the dark for 2 h and subjected to ion-exchange chromatography and gel filtration under the conditions described above for the irradiated reaction mixture.

Isolation of the Cross-Linked Peptide. Protein samples (15 mg) from each of the pooled and lyophilized fractions A, B, and C (Figure 4) were oxidized with performic acid (Hirs, 1967) and treated with trypsin (0.3 mg) in 0.1 M ammonium bicarbonate (3 ml) at 37 °C for 4 h. The lyophilized tryptic peptides mixtures were subjected to paper electrophoresis at pH 1.9 (3 kV for 30 min). The paper was thoroughly dried by a stream of cold air, and longitudinal strips (1.5-cm wide) were cut from both sides of the paper. These were developed by ninhydrin–cadmium reagent (Dreyer and Bynum, 1967) and then cut into horizontal strips (1.5 cm) which were counted in toluene to identify radioactive bands. The radioactive and ninhydrin-positive bands were cut out of the remaining paper, stitched to another sheet of Whatman No. 3 MM paper, and subjected to descending chromatography in 1-butanol–acetic acid–water–pyridine (15:3:12:10, v/v) for 25 h. Cold and radioactive peptides were located as described above. The radioactive and ninhydrin-positive bands were cut out and eluted with 0.2 M ammonia solution. The solvent was evaporated and the remaining peptide was acid hydrolyzed for amino acid analysis.

Results

Irradiation of RNase and [^{14}C]pUp or [^{14}C]pCp. The time course for incorporation of pCp and pUp into RNase is shown in Figure 1, where % incorporation = (moles of nucleotide/mole of RNase)100. Within 4 h up to 50% incorporation of labeled nucleotides into RNase could be detected. After that time the incorporation reached a plateau which was accompanied by the loss of RNase activity as will be shown below.

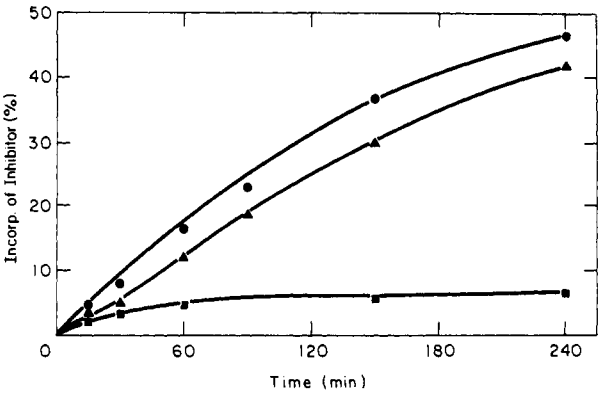

FIGURE 1: Time course for incorporation of radioactive pCp and pUp into RNase. Mixtures of the enzyme and the inhibitors were irradiated with light of $\lambda > 300$ nm (Pyrex filter) in the presence of acetone, as described under Methods. Samples were withdrawn at various time intervals, lyophilized, dissolved in 20 mM ammonium bicarbonate, and loaded onto a column (2 × 0.5 cm) of Dowex 2 × 10 (HCO_3^- form) equilibrated with the same buffer. The modified and nonmodified RNase were eluted with 20 mM ammonium bicarbonate. The amount of protein in the break through fractions was determined by amino acid analysis, and the radioactivity incorporated was monitored as described under Methods. (●—●) RNase cross-linked to pUp; (▲—▲) RNase cross-linked to pCp; (■—■) performic acid oxidized RNase cross-linked to pUp.

Irradiation of Denatured RNase and pUp. Performic acid oxidized RNase (Hirs, 1967) was irradiated in the presence of [^{14}C]pUp, and the incorporation of radioactive label was followed in order to determine the extent of nonspecific cross-linking. The results (Figure 1) indicate that only 6% of pUp was incorporated into oxidized RNase as compared with a maximum of 50% incorporation into the native enzyme under the same irradiation conditions.

Inhibition of [^{14}C]pUp Cross-Linking by 3′-UMP. The fact that denatured RNase cross-links very poorly to pUp suggests that the cross-linking is specific and involves only bound molecules. In order to establish this result and to show that binding of pUp to RNase occurs at the site for pyrimidine nucleotide binding (Richards and Wyckoff, 1971), we irradiated mixtures of the enzyme and [^{14}C]pUp and followed incorporation of radioactivity in the presence of increasing amounts of cold 3′-UMP. The results (Figure 2) indicate that 3′-UMP reduces the amount of [^{14}C]pUp incorporation into the enzyme. Practically total inhibition is achieved in the presence of about 20-fold excess of 3′-UMP.

Effect of Irradiation on RNase Activity. Dark incubation of the enzyme in a buffer containing 5% acetone does not affect its activity at all; irradiation with ultraviolet light of $\lambda > 260$ nm results in a fast decrease of the initial activity of the enzyme. This irradiation-induced inactivation of RNase can, however, be reduced by employing acetone and light of $\lambda > 300$ nm (see Figure 3). An optimal irradiation period of 1 h is therefore chosen for the experiments aiming at isolation of labeled peptides. During this period enough label is incorporated into the enzyme (15 and 10% with pUp and pCp, respectively), while the reaction mixture still contains 40% of active enzyme.

Characterization of the Cross-Linked Product. I. Irradiation. A standard reaction mixture (3 ml) of either

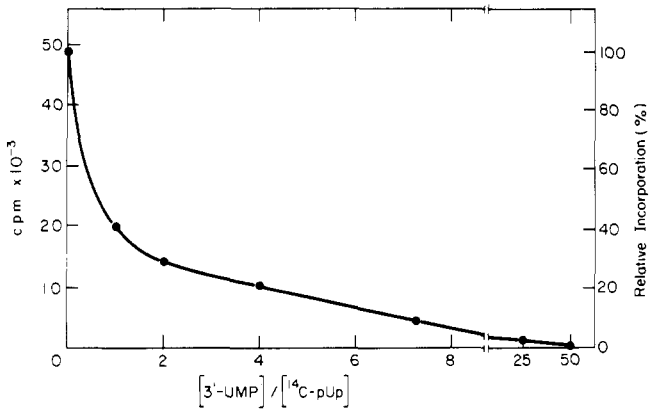

FIGURE 2: Inhibition of [^{14}C]pUp cross-linking, induced by 3′-UMP Standard mixtures of RNase and [^{14}C]pUp (0.3 ml each) containing increasing amounts of cold 3′-UMP were irradiated for 1 h. The incorporation of radioactivity into the protein was determined as described in Figure 1.

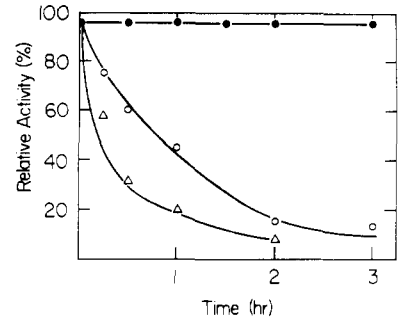

FIGURE 3: Irradiation-induced inactivation of RNase. A solution of RNase (1 ml of 0.7 mM enzyme in 20 mM sodium acetate buffer pH 5.5) was irradiated and its enzymatic activity was assayed as described under Methods. (●—●) RNase incubated in the dark in buffer containing 5% acetone; (○—○) RNase irradiated with ultraviolet light of λ >300 nm (Pyrex filter) in the presence of 5% acetone; (△—△) RNase irradiated with light of λ >260 nm (Corex filter).

[^{14}C]pUp or [^{14}C]pCp was irradiated for 1 h (λ >300 nm) in the presence of 5% acetone. Ion-exchange chromatography of the reaction products indicated that 15 and 10% of cross-linking occurred with pUp and pCp, respectively. Reaction mixtures incubated in the dark were subjected to the same separation procedure described above. In this case the enzyme, which was totally recovered in the breakthrough fractions, contained no radioactivity. The radioactive nucleotides were recovered completely by applying the usual ammonium bicarbonate gradient.

II. Gel Filtration. The protein fraction isolated above was subjected to gel filtration on Sephadex G-50 superfine column equilibrated and eluted with 0.1 M acetic acid. The elution profile is shown in Figure 4. Amino acid analyses of aliquots from peaks A, B, and C gave amino acid compositions almost identical with that of native RNase. Fraction D contained no amino acids and had the same elution volume on Sephadex G-50 and the same electrophoretic mobility on paper at pH 1.9 as those of uridine.

III. Gel Electrophoresis. Fraction C, as gel filtration indicated (Figure 4), was the same size as native RNase, and had the same elution profile as the dark reaction product. Since A and B, as well as C, had the amino acid composition of RNase, it was attempted to estimate the molecular weights of these products, which seem to result from protein–protein cross-linking. Samples (10 μg) of each of fractions A, B, and C, in 0.1 M phosphate buffer (pH 7.2), 0.1% sodium dodecyl sulfate, were analyzed on 10% polyacrylamide gels (in 0.1% sodium dodecyl sulfate) which were calibrated with standards of known molecular weights (Weber and Osborn, 1969).

The mobilities of fractions C and B corresponded to species of molecular weight of 14 000 and 28 000 (±5%) and will be referred to below as the monomer and dimer, respectively The major band in A exhibited a molecular weight of 39 000 (trimer), and in addition a weak band of tetramer and traces of bands of higher aggregates were present. Scanning the sodium dodecyl sulfate gels for radioactivity revealed that all the protein bands were also radioactive, similar to the gel filtration results. This indicates that the inhibitors were indeed covalently bound to the enzyme in the monomer, as well as in the higher aggregates. It should be noted that, in experiments initiated with light of λ >260 nm, all cross-linked material was present as dimers and higher aggregates. However, in the photosensitized experiments, as Figure 4 indicates, approximately 40% of monomeric material could be recovered.

IV. Isolation and Identification of the Cross-Linked Labeled Peptide in the Monomer. Purified fraction C from the RNase–pUp reaction was performic acid oxidized and digested with trypsin as described in Methods. High-voltage paper electrophoresis at pH 1.9 revealed a single radioactive and ninhydrin-positive band with mobility of 0.21 with respect to aspartic acid. Chromatography in the second dimension (Figure 5) separated this band into two major ninhydrin-positive bands (R_f 0.34 and R_f 0.50 (relative to uridine)), only the first one of which was radioactive, and a third weak band (R_f 1; uridine, being 1), which was radioactive and ninhydrin negative. The third band had the same mobility as uridine, it gave a positive reaction for reducing sugars (Stahl, 1969) and a negative reaction for organic phosphates (Hanes and Isherwood, 1949). It was thus identified as uridine which could be formed as a result of partial decomposition of the products. Similar treatment of the monomer fraction from the reaction of RNase and pCp gave similar results on electrophoresis and paper chromatography. In this case one radioactive and ninhydrin-positive spot was obtained and traces of cytidine could be identified in the chromatogram.

The amino acid compositions of the radioactive peptides eluted from the paper are given in Table I. A blank experiment omitting the nucleotides was also performed. RNase was irradiated and underwent the same procedure as described for the labeled enzyme. The peptide which had the same electrophoretic and chromatographic behavior as those of the labeled peptides was isolated and its amino acid composition is given in Table I. The amino acid compositions of these peptides are consistent with the sequence Asn-67-Arg-85 of RNase. The specific activity, however, was 30 and 10% of that expected for a 1:1 cross-linked peptide with pUp and pCp, respectively. This can be explained by the fact that both labeled and unlabeled peptide Asn-67-Arg-85 behave identically in paper electrophoresis and chromatography, as indeed was shown above. The similarity of the electrophoretic mobilities of the labeled and unlabeled peptides can be explained by assuming that the phosphate groups were cleaved off during the cross-linking process, or due to the subsequent treatment of the cross-linked protein. This would leave a neutral label on the peptide. The appearance of uridine in the final chromatographic step supports this assumption. The verification of this point must however

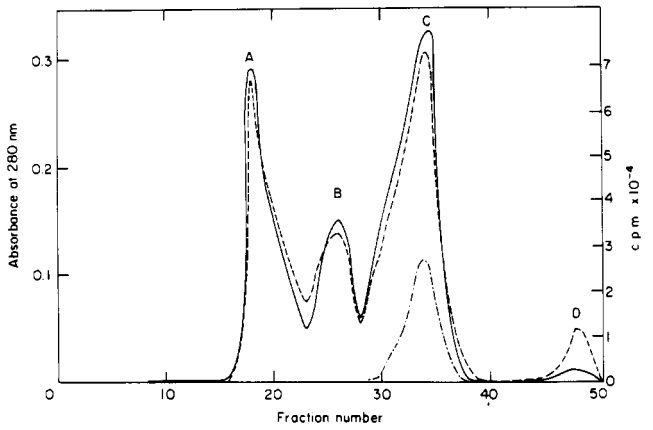

FIGURE 4: Elution profile of RNase cross-linked to pUp from a column (130 × 2.8 cm) of Sephadex G-50 superfine equilibrated and eluted with 0.1 M acetic acid. Fractions of 6 ml were collected at a flow rate of 25 ml/h. (—) Absorbance at 280 nm; (- - -) total radioactivity; (- · -) absorbance at 280 nm of RNase isolated from the blank dark reaction, no radioactivity was associated with this peak. The fractions corresponding to peaks A–D were pooled and further treated separately.

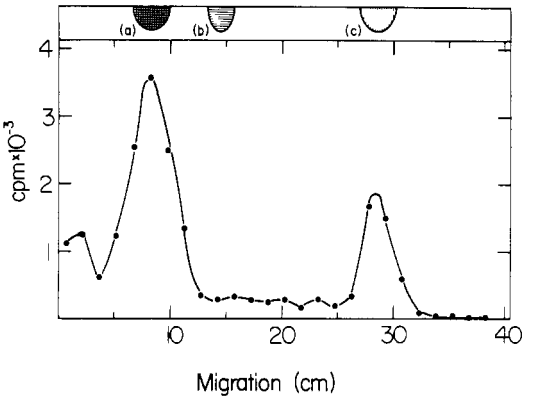

FIGURE 5: Chromatographic pattern of the radioactive band which was obtained from the tryptic digest of pUp-labeled monomer, by electrophoresis at pH 1.9. The radioactive band was stitched to Whatman No. 3MM paper and chromatographed in 1-butanol–acetic acid–water–pyridine (15:3:12:10, v/v) for 25 h. Strips were cut out, developed with ninhydrin–cadmium reagent and assayed for radioactivity as described under Methods. Upper pattern: (a) radioactive and ninhydrin-positive band, identified as peptide Asn-67-Arg-85; (b) ninhydrin-positive band; (c) radioactive band identified as uridine.

await more detailed analyses of the cross-linked products and the chemical mechanism of their formation. The possibility that a radioactive by-product of the reaction was coincidentally attached to peptide 67–85 was, however, ruled out by showing that none of the irradiation products of either pCp or pUp co-migrated with peptide 67–85.

The labeled dimers, trimers, and higher aggregates were processed in the same manner as the monomer, i.e., trypsin digest, electrophoresis, and chromatography. In these cases, also, only one peptide was both radioactive and ninhydrin positive. However, the amino acid compositions of labeled peptides obtained from the dimers and higher aggregates could not be interpreted in terms of a single peptide chain even after further purification. These products, most probably, contained cross-linked peptides which result from protein–protein cross-linking as described in the Discussion. The major component of these cross-linked peptides was still consistent with peptide 67–85.

Irradiation of pCp and pUp. The nucleoside diphosphates, in 20 mM acetate buffer pH 5.5, were irradiated in the presence of acetone. The reaction mixtures were analyzed by paper electrophoresis at pH 1.9. In each experiment two radioactive spots were revealed, the first corresponded to starting materials (pUp, $R_f - 1.1$; pCp, $R_f - 0.5$ relative to aspartic acid). The second ($R_f - 1.9$ and $R_f - 1$ relative to aspartic acid in the pUp and pCp experiments, respectively) exhibited no fluorescent spot on the paper, but gave a positive phosphate reaction; it was thus identified as a mixture of pyrimidine diphosphate dimers (Elad et al., 1971).

Table I: Amino Acid Composition of the Tryptic Radioactive Peptides Isolated from RNase Covalently Labeled with pCp or pUp.

Amino Acid	Residues[a] pCp Labeled Peptide	pUp Labeled Peptide	Tryptic Peptide 67–85[b]	Tryptic Peptide 67–85 Theor[c]
Lysine	0.22	0.15	0.22	
Arginine	0.66	0.7	1.03	1
Aspartic acid	3	3	3	3
Threonine	2.45	2.54	2.88	3
Serine	2.6	2.94	2.78	3
Glutamic acid	2.2	2.2	2.2	2
Glycine	1.1	1.09	1.3	1
Alanine	0.29	0.09	0.1	
Cysteic acid	1.95	1.92	2.02	2
Methionine sulphone	0.91	0.92	1.07	1
Isoleucine	0.7	0.87	0.92	1
Tyrosine	1.86	1.8	2.16	2
Specific activity[d]	0.3	0.1		

[a] Based on three residues of aspartic acid per peptide. [b] Isolated from a tryptic digest of performic acid oxidized irradiated RNase prepared by the procedure described for the pUp or pCp labeled enzyme. This peptide had the same mobilities, in both electrophoresis and chromatography, as those of the labeled peptides. [c] According to Smyth et al. (1963). [d] Expressed as moles of nucleotide per mole of peptide.

Discussion

The irradiation-induced cross-linking of pyrimidine nucleotides to RNase took place at pH 5.5, where the association constant for these complexes reaches a maximum value of ca. 10^6 M^{-1} (Sawada and Irie, 1969; Gorecki, 1971). It could be induced either by direct irradiation with ultraviolet light of $\lambda > 260$ nm, or through photosensitization with acetone and light of $\lambda > 300$ nm. In the first case, where both the nucleotide and the enzyme absorb the incident light, the desired protein–nucleotide cross-linking is accompanied by a considerable amount of protein–protein cross-linking and destruction of light-sensitive amino acids, which complicate the analyses of the cross-linked fragments. In the photosensitized reaction, however, most of the incident light is absorbed by acetone, and the excitation energy is transferred rather efficiently to the nucleotide (Elad et al., 1971). Although the detailed mechanism of the cross-linking reaction is still unknown, it is feasible that excited nucleotide molecules, which are bound to the enzyme, cross-link with neighboring amino acid residues to form the specific adducts. Free excited nucleotide molecules prefer, however, to

revert to ground state or react with ground state nucleotide to yield the known cyclobutane dimers of the pyrimidine derivatives. Energy transfer to amino acid residues on the protein might also occur, with less efficiency, however. This might subsequently lead to the generation of free radicals on the protein (Sperling and Elad, 1971; Sperling, 1971), which will eventually cause protein–protein cross-linking, in addition to nucleotide–protein cross-linking. It should be pointed out that acetone does not inactivate RNase in the absence of ultraviolet light. The long wavelength irradiation in the photosensitized reaction results in a slower rate of inactivation of the enzyme and induces less destruction of light-sensitive amino acid residues than the direct irradiation does. We, therefore, prefer the photosensitization method to induce protein to nucleotide cross-linking since it allows a better and unambiguous interpretation of results, especially amino acid analyses. Our experiments in cross-linking of histone H4 and ATP (Sperling, 1976) indicate that, by the use of photosensitization, a monomeric cross-linked protein could be obtained in yields of 50%.

The specificity of cross-linking has been demonstrated by showing that a single peptide in RNase became covalently cross-linked to both nucleotides, whereas the denatured enzyme failed to cross-link specifically with the inhibitors. Furthermore, the presence of a large molar excess of nucleotide in the reaction mixtures did not lead to nonspecific cross-links. Concerning the location of the cross-link, it should be pointed out that there is one binding site on RNase with a high association constant for pyrimidine nucleotides (Richards and Wyckoff, 1971). pCp and pUp bind strongly to RNase and competitively inhibit its hydrolytic activity toward cytidine 2′,3′-cyclic phosphate with $K_I = 10^{-6}$ M (Sawada and Irie, 1969; Gorecki, 1971). We have further demonstrated the competitive nature of pUp binding by showing that the presence of increasing amounts of 3′-UMP in the irradiation mixtures caused a sharp decrease in the extents with which radioactive pUp became cross-linked to RNase. It can be concluded, therefore, that pCp and pUp occupy the same position as 3′-UMP in the tertiary structure of their complexes with RNase. The amino acid composition of the peptide labeled either with [^{14}C]pUp or [^{14}C]pCp was consistent with the sequence of tryptic peptide Asn-67-Arg-85 of performic acid oxidized RNase. The amino acid analyses of the labeled peptides gave relatively low values for Ile, Thr, Ser, and Arg. It should be noted that, in the region consisting of residues 78 through 82, which is at the bottom of the site for pyrimidine binding (see below), there are two Ser residues (77 and 80), two Thr residues (78 and 82), and the only Ile residue of this peptide (81). It can be speculated that these residues are suitable candidates for being modified by cross-linking to the pyrimidine ring. This point, however, needs further proof.

The significance of pUp and pCp binding covalently to peptide 67–85 is difficult to assess. The pyrimidine moiety of nucleotides has been shown to occupy a groove, bounded in part by residues Thr 45, Phe 120, and Ser 123, which has been designated the B_1 site (Wyckoff, 1968; Richards and Wyckoff, 1971; Meadows et al., 1969). The sequence Asn-67-Arg-85 is part of peptide 71–92 which together with peptide 94–110 constitute a twisted antiparallel pleated sheet structure (Kartha et al., 1967; Richards and Wyckoff, 1973). This structure is bent and joins the two wings of the RNase molecule. The pyrimidine ring, which is bound at the B_1 site of the enzyme, points in the direction of the apice of the V-shaped molecule and, thus, is close to residues 77–82 which are at the bottom of site B_1. Therefore, it seems likely that one of these residues is involved in the photochemical cross-linking.

Acknowledgments

We thank Professor Dov Elad for his continued interest and encouragement, Dr. Yigal Burstein for his useful suggestions, and Dr. Ruth Sperling for her helpful comments on the manuscript.

References

Anfinsen, C. B. (1954), *J. Biol. Chem. 207,* 201.
Ben-Ishai, R., Green, M., Graff, E., Elad, D., Steinmaus, H., and Salomon, J. (1973), *Photochem. Photobiol. 17,* 155.
Braun, A., and Merrick, B. (1975), *Photochem. Photobiol. 21,* 243.
Bray, G. A. (1960), *Anal. Biochem. 1,* 279.
Budzik, G. P., Lam, S. S. M., Schoemaker, H. J. P., and Schimmel, P. R. (1975), *J. Biol. Chem. 250,* 4433.
Cooperman, B. S., and Brunswick, D. J. (1973), *Biochemistry 12,* 4079.
Dreyer, W. J., and Bynum, E. (1967), *Methods Enzymol. 11,* 32.
Elad, D., Rosenthal, I., and Sasson, S. (1971), *J. Chem. Soc. C,* 2053.
Gorecki, M. (1971), Ph.D. Thesis, The Feinberg Graduate School, The Weizmann Institute of Science, Rehovot, Israel.
Gorelic, L. S., Lisagor, P., and Yang, N. C. (1972), *Photochem. Photobiol. 16,* 465.
Hall, R. E., and Khorana, H. G. (1955), *J. Am. Chem. Soc. 77,* 1871.
Hanes, C. S., and Isherwood, F. A. (1949), *Nature (London) 164,* 1107.
Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 197.
Jellinek, T., and Johns, R. B. (1970), *Photochem. Photobiol. 11,* 349.
Kartha, G., Bello, J., and Harker, D. (1967), *Nature (London) 213,* 862.
Kurland, C. G. (1972), *Annu. Rev. Biochem. 41,* 377.
Lin, S.-Y., and Riggs, A. D. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 947.
Markovitz, A. (1972), *Biochim. Biophys. Acta 281,* 522.
Meadows, D. H., Roberts, G. C. K., and Jardetzky, O. (1969), *J. Mol. Biol. 45,* 491.
Richards, F. M., and Wyckoff, H. W. (1971), *Enzymes, 3rd Ed. 4,* 467–806.
Richards, F. M., and Wyckoff, H. W. (1973), in Atlas of Molecular Structures in Biology, Phillips, D. C., and Richards, F. M., Ed., Oxford, Clarendon Press, p 1.
Sawada, F., and Irie, M. (1969), *J. Biochem. (Tokyo) 66,* 415.
Schoemaker, H. J. P., Budzik, G. P., Giegé, R., and Schimmel, P. R. (1975), *J. Biol. Chem. 250,* 4440.
Schoemaker, H. J. P., and Schimmel, P. R. (1974), *J. Mol. Biol. 84,* 503.
Sela, M., and Anfinsen, C. B. (1957), *Biochim. Biophys. Acta 24,* 229.
Smith, K. C. (1964), *Photochem. Photobiol. 3,* 415.
Smith, K. C. (1975), in Photochemistry and Photobiology of Nucleic Acids, Wang, S. Y., Ed., New York, N.Y., Academic Press.
Smith, K. C., and Meun, D. H. C. (1968), *Biochemistry 7,*

1033.
Smyth, D. G., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238,* 227.
Sperling, J. (1971), *J. Am. Chem. Soc. 93,* 3839.
Sperling, J. (1976), *Photochem. Photobiol. (in press).*
Sperling, J., and Elad, D. (1971), *J. Am. Chem. Soc. 93,* 967.
Stahl, E. (1969), Thin Layer Chromatography, New York, N.Y., Springer-Verlag, p 899.
Varghese, A. J. (1974), *Photochem. Photobiol. 20,* 339.
von Hippel, P. H., and McGhee, J. D. (1972), *Annu. Rev. Biochem. 41,* 231.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Weintraub, H. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 247.
Wyckoff, H. W. (1968), *Brookhaven Symp. Biol. 21,* 252.
Wyckoff, H. W., Tsernoglou, D., Hanson, A. W., Knox, J. R., Lee, B., and Richards, F. M. (1970), *J. Biol. Chem. 245,* 305.

The Nature of Stacking Interactions in Polynucleotides. Molecular States in Oligo- and Polyribocytidylic Acids by Relaxation Analysis†

Dietmar Pörschke

ABSTRACT: The dynamics of the helix–coil transition of single-stranded poly(C) (polyribocytidylate) and CpC (cytidylyl(3′-5′)cytosine) was investigated by an improved cable temperature-jump technique. The single-strand relaxation was characterized by following the ultraviolet (uv) absorbance changes at 248 and 280 nm. Poly(C) and CpC showed single relaxation processes with amplitudes corresponding to those expected from equilibrium melting curves. The relaxation time constants in the range of 25–100 ns were independent of the nucleotide concentration, but strongly dependent upon temperature. Using thermodynamic parameters obtained from circular dichroism (CD) and uv absorbance melting curves, the following rate constants k (at 20 °C, 1.05 M ionic strength, pH 7) and activation enthalpies E_A were calculated for poly(C): helix formation $k_R = 1.11 \times 10^7\ s^{-1}$ ($E_{AR} = 2.6$ kcal); helix dissociation $k_D = 2.1 \times 10^6\ s^{-1}$ ($E_{AD} = 11.9$ kcal). The rate constants obtained for CpC were higher by a factor of about 2 in k_R and 12 in k_D, whereas the activation enthalpies closely corresponded to those found for the polymer. In addition to the single-stranded helix–coil relaxation, poly(C) and CpC exhibit a relaxation process with a time constant below 25 ns and maximum amplitudes at wavelengths $\lambda \geq 285$ nm. The same process is found in cytidine and is attributed to hydration equilibria. The hydration reaction can be considered to be in equilibrium during the entire time range of the helix–coil transition and thus the data obtained for the helix–coil transition can be described by a simple two-state model. The rate parameters indicate the existence of relatively high energy barriers in the helix–coil transition and provide strong evidence against an oscillating dimer model. If there is an ensemble of substates for one of the states (as may be expected for the coil form), the energy difference between the populated substates is small compared with the energy difference between the major conformational states.

The conformation of single-stranded polynucleotides has been studied by nearly all the methods available for the investigation of macromolecular structures. Measurements of uv absorbance, ORD, CD, NMR, light scattering and various other parameters have led to the conclusion that some polynucleotides, like poly(A) and poly(C)[1], exist in a single-stranded helical form with the bases stacked upon each other (Felsenfeld and Miles, 1967; Ts'o, 1974; Bloomfield et al., 1974). The main driving force for the formation of the helix is the stacking interaction between adjacent bases. Data collected for oligonucleotides of various chain lengths showed that the formation of the single-stranded helix is almost uncooperative; i.e., the stacking interaction of two adjacent monomer units is almost independent of the state of their neighbors, and the main interactions leading to the formation of the helix are between consecutive RNA residues (cf. Brahms et al., 1967). A scheme of the single-stranded helix and the coil form is given in Figure 1.

The thermodynamics of the helix–coil transition is usually described by a simple two-state model. However, the validity of the two-state model has been questioned for several reasons and a dynamic structure has been proposed, in which the bases oscillate with respect to one another (Glaubiger et al., 1968; Davis and Tinoco, 1968). Various arguments have been used in favor of one or the other model. However, a clear distinction was not possible on the basis of the available data. This uncertainty simply is due to the fact that all the methods applied hitherto, including NMR, only give an average picture of the polymer conformations since the lifetime of individual conformational states is much shorter than the time resolution of the techniques applied. In order to learn about the number of conformational states and the type of coupling between them, the time resolution of the analyzing technique has to be sufficiently high. In the

† From the Max Planck Institut für biophysikalische Chemie, D-3400 Göttingen, Germany. *Received August 27, 1975.*

[1] Abbreviations used: CpC, cytidylyl (3′-5′)cytosine; poly(C), polyribocytidylate; CMP, cytidine 5′-monophosphate; poly(A), polyriboadenylate.