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Specificity of Photochemical Cross-Linking in Protein-Nucleic Acid Complexes: Identification of the Interacting Residues in RNase-Pyrimidine Nucleotide Complex†

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ABSTRACT: This report presents evidence in favor of the supposition that photochemical cross-linking between the partners of nucleoprotein complexes is specific and involves only residues which are in close proximity in the native structure. RNase was covalently linked to its ^{14}C-labeled competitive inhibitor uridine 2′(3′),5′-diphosphate (pUp) by ultraviolet irradiation of the enzyme-inhibitor complex. The amino acid residues which took part in the photochemical reaction were identified as Ser-80, Ile-81, and Thr-82. Ion-exchange chromatography of the irradiated complex afforded modified RNase containing one covalently bound molecule of [^{14}C]pUp (RNase-pUp). A tryptic digest of the performic acid oxidized RNase-pUp gave a single labeled peptide whose amino acid composition was consistent with the sequence Asn-67-Arg-85. Further degradation with thermolysin gave five peptides, only two of which were radioactive. One of these, Thr-76-Arg-85, contained one molecule of the labeled inhibitor and was totally deficient in Ser-80 and Ile-81. In the second, Ile-81-Arg-85, half of Thr-82 was missing and it contained half an equivalent of bound inhibitor. It is suggested that the initial attack of the excited pUp occurred at Ser-80 with subsequent modification of Ile-81. Thr-82 was modified, most probably, by a different mechanism to give a labile, though covalent, addition product. The pyrimidine ring of the nucleotide in the enzyme-inhibitor complex points in the direction of peptide 78-82 which constitutes the bottom of the binding site for the pyrimidine ring (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). Thus, the identification of Ser-80, Ile-81, and Thr-82 as the residues which cross-linked photochemically to pUp indicates that such cross-linking is specific and involves only neighboring groups in the native structure of the nucleoprotein complex.

The tendency of proteins and nucleic acids to form stable covalent complexes as a result of ultraviolet irradiation has increasingly been used as a probe for studying the structure of native nucleoprotein complexes. Recent examples of problems which were studied by this approach are the interactions between aminoacyl-tRNA synthetases and their cognate tRNAs (Schoemaker et al., 1975; Budzik et al., 1975), ribosomal proteins and ribosomal RNA (Gorelic, 1976), coat protein and RNA in bacteriophage MS2 (Budowsky et al., 1976), and the interactions between DNA and histones in chromatin (Strniste and Rall, 1976; Sperling and Sperling, 1977). These studies are based on the assumption that the UV[1]-induced covalent cross-links are formed between interacting regions on the macromolecules, or between residues which are in close proximity in the native complex. The compliance with this condition would mean that the photochemical cross-linking "freezes" existing contact points in the irradiated protein-nucleic acid complex and, thereby, allows the identification of the interacting regions by chemical means. It is apparent, therefore, that the reliability of the photochemical approach depends on the ability of both purines and pyrimidines to form covalent adducts with a major number of amino acids—without particular preference toward specific ones. At the same time it requires that specific covalent bonds would be formed only between neighboring residues in the native structure.

The photochemical induction of addition products between pyrimidines and amino acids has been reported in several publications (Jellinek and Johns, 1970; Gorelic et al., 1972; Varghese, 1974; Sawada, 1975; Sperling and Havron, 1976). Recently, it has also been shown that stable covalent cross-links occur upon irradiation of complexes of various proteins and purine nucleoside triphosphates or cyclic phosphates (Sperling, 1976; Antonoff et al., 1976). As far as amino acids are concerned it has been shown, mainly by using low molecular weight model compounds, that a large variety of amino acids takes part in cross-linking to uracil and thymine derivatives (Smith, 1969; Schott and Shetlar, 1974; Shetlar et al., 1975).

In our previous work (Sperling and Havron, 1976) we have photochemically linked RNase A to two of its competitive inhibitors—pUp and pCp. We have shown, by three different criteria, that the cross-linking was specific: (a) the denatured enzyme failed to cross-link with the inhibitors; (b) the extent of covalent binding of pUp could be reduced by the addition of increasing amounts of another competitive inhibitor (3′-

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[1] Abbreviations used are: pUp, uridine 2′(3′),5′-diphosphate; pCp, cytidine 2′(3′),5′-diphosphate; 3′-UMP, uridine 3′-monophosphate; HVPE, high voltage paper electrophoresis; DBAE-cellulose, *N*-[*N*′-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose; UV, ultraviolet; IR, infrared; TLC, thin-layer chromatography.

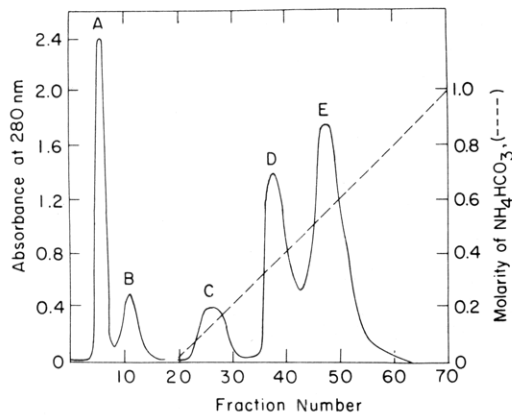

FIGURE 1: Elution profile of a photochemical cross-linking reaction mixture of RNase with [^{14}C]pUp from a column (40 × 1.8 cm) of Dowex 2-X10 (bicarbonate form). A linear gradient (0.02–1.0 M) of NH_4HCO_3 was applied as indicated at a flow rate of 2 mL/min. Fraction A is nonmodified RNase. Fraction C is RNase which incorporated 1 mol of [^{14}C]pUp per mol of enzyme (RNase–pUp). Fractions B, D, and E are uridine, 3′(5′)-UMP, and pUp, respectively.

UMP); and (c) a single tryptic peptide (Asn-67-Arg-85) of RNase became covalenty linked to both pUp and pCp even in the presence of a large excess of either inhibitor.

In this publication we report the identification of the amino acid residues Ser-80, Ile-81, and Thr-82 of RNase as those participating in the covalent binding of the enzyme to pUp. These residues, as the three-dimensional model of RNase reveals, are in close proximity to the pyrimidine ring of pUp in the enzyme–inhibitor complex (Richards and Wyckoff, 1973), and thus would be expected to cross-link to the inhibitor.

Experimental Section

Irradiations were carried out in a Wild Universal unit (Wild Heerbrugg, Switzerland) with a 200-W super pressure mercury lamp (HBO 200W, Osram, West Germany). The sample, in a 3-mL spectrophotometric cell, was placed at a distance of 15 cm from the light source in an ice–water-cooled jacket and maintained at 25 °C. IR radiation and UV radiation of wavelengths shorter than 300 nm were eliminated by introducing into the light path a Pyrex cell of 2-cm path length filled with distilled water.

The light intensity was 2.1×10^{-6} einstein min^{-1} cm^{-2}. It was determined by benzophenone–benzhydrol actinometry (Wagner, 1969) as described by Rosenthal and Bercovici (1976). The reaction to be described is photosensitized by acetone and light of $\lambda > 300$ nm (Pyrex filter) which is absorbed by acetone. Therefore, the efficient light intensity was determined by subtracting the intensity which was transmitted through the reaction mixture from the total intensity in the range of 300–400 nm.

Preparation and Purification of RNase Containing One Covalently Bound Molecule of [^{14}C]*pUp* (*RNase–pUp*). A solution (12 mL), 6.17 mM in RNase, 19.5 mM in [U-^{14}C]-pUp (specific activity 445 cpm/nmol), and 50 mM in sodium acetate–acetic acid buffer (pH 5.5) containing 5% (v/v) acetone was irradiated (4 × 3 mL portions) for 1 h. The lyophilized reaction mixture was dissolved in 20 mM NH_4HCO_3 (10 mL) and applied onto a column (40 × 1.8 cm) of the bicarbonate form of Dowex 2-X10, equilibrated with the same buffer. Unreacted enzyme was eluted with the starting buffer. Cross-linked RNase and unreacted inhibitor were subsequently eluted by a linear gradient (0.02–1.0 M) of NH_4HCO_3, at a flow rate of 2 mL/min (see Figure 1). The protein containing fractions (A and C) were pooled, lyophilized, and subjected to gel filtration on Sephadex G-50 superfine column (110 × 2.9 cm) equilibrated and eluted with 0.1 M acetic acid.

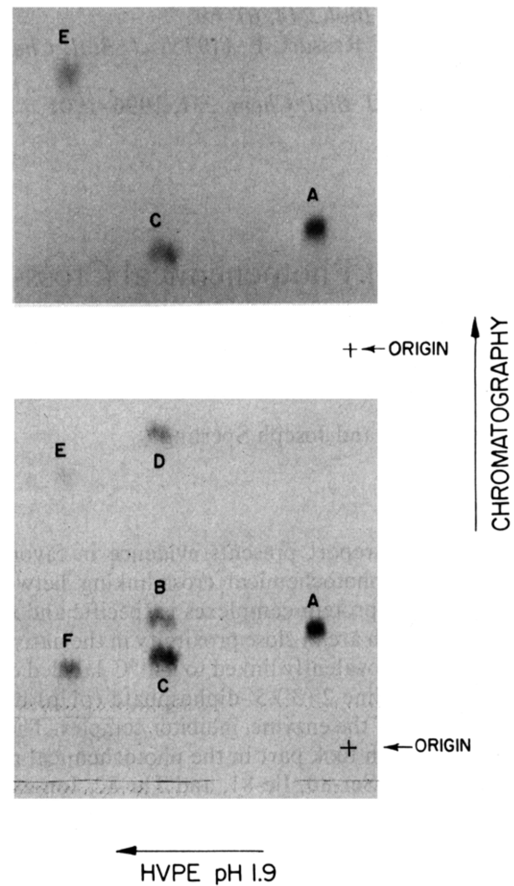

FIGURE 2: Two-dimensional separation on paper of a thermolysin digest of cross-linked tryptic peptide Asn-67-Arg-85 of RNase. The peptide (20 nmol) was digested with thermolysin and fingerprinted as described in the Experimental Section. Lower plate: ninhydrin stain pattern. Upper plate: radioactivity pattern.

Isolation of cross-linked peptide (*Asn-67-Arg-85*) was carried out as described previously (Sperling and Havron, 1976) by two-dimensional separation on paper of the tryptic digest of performic acid oxidized RNase–pUp.

Thermolysin Digestion of Cross-Linked Peptide 67–85. The peptide (400 nmol) was dissolved in NH_4HCO_3 solution (0.6 mL of 0.5%, pH 8.1), and incubated with purified thermolysin (10 μg) at 55 °C for 6 h. [Thermolysin (Protease type X) was purchased from Sigma and purified by gel filtration on Sephadex G-50 fine.]

Separation and Identification of Thermolytic Peptides from Cross-Linked Peptide 67–85. A volume of the above reaction mixture corresponding to 150 nmol of peptide 67–85 was loaded on Whatman No. 3MM paper (20 nmol/cm) alongside with three 20 nmol control and marker portions—each spotted on 1 cm. The entire paper was subjected to HVPE (60 V/cm) at pH 1.9 for 25 min. A paper strip of one control sample was removed, stained with ninhydrin-cadmium reagent, and cut into horizontal strips (1.5 cm wide) which were counted in toluene scintillation liquid. A second control strip was stitched onto a sheet of Whatman No. 2 paper (prewashed with 0.5 M acetic acid) and subjected to descending chromatography in butanol:acetic acid:water:pyridine mixture (15:3:10:12) for 17 h. The dried paper was stained with ninhydrin–cadmium reagent and autoradiographed for 48 h to produce the peptide fingerprint shown in Figure 2.

Horizontal strips, corresponding to the ninhydrin positive

and radioactive bands of the first control, were cut out from the remaining paper containing the third control and the 150-nmol samples. Each of these was stitched to a Whatman No. 3MM paper and chromatographed as above. The markers of the third control sample were stained with ninhydrin and their radioactivity was monitored. The separated peptide bands were eluted from the remaining paper with 0.5 M acetic acid, and their amino acid composition was determined after hydrolysis in 6 N HCl.

Separation of Labeled Amino Acids and Small Peptides by Affinity Chromatography on DBAE-Cellulose. (a) Alkaline Phosphatase and Pronase Digestions. Cross-linked peptide Asn-67-Arg-85 (0.1 μmol) was dissolved in 0.5% NH_4HCO_3, pH 8.1 (0.5 mL). Alkaline phosphatase (Worthington, *E. coli*, 10 μL of 5 μg/mL) was added and the mixture was incubated at 37 °C for 3 h. The lyophilized sample was redissolved in 0.5% NH_4HCO_3, pH 8.1 (0.3 mL). Pronase (Calbiochem, 5 μL of 1 mg/mL in 1 mM HCl) was added; the solution was incubated at 37 °C for 5 h and then lyophilized.

(b) DBAE-Cellulose Affinity Chromatography. The digested peptide (60 nmol, 27 000 cpm) was dissolved in buffer A [0.3 mL of 0.05 M *N*-methylmorpholine, pH 7.7, 0.6 M KCl, 20% ethanol (v/v)] and loaded at 4 °C on a column (0.9 × 6 cm) of acetylated DBAE-cellulose (Collaborative Research Inc.) equilibrated with the same buffer. Elution with buffer A (20 mL) yielded nonmodified fragments of peptide 67-85. Subsequent elution with buffer B (20 mL of 0.05 M sodium acetate (pH 5.0), 0.2 M NaCl) afforded the modified radioactive fragments (see Figure 3). The retarded fraction was subjected to amino acid analysis after acid hydrolysis.

Results

Preparation and Properties of the 1:1 Covalent Adduct of RNase and pUp. The complex of RNase and [^{14}C]pUp was irradiated in the presence of acetone as described before (Sperling and Havron, 1976). The whole reaction mixture was subjected to ion-exchange chromatography on Dowex 2-X10 using a linear gradient of NH_4HCO_3 as eluent (Figure 1). The starting buffer eluted nonmodified RNase (98% of the starting enzyme, fraction A) which was fully active. Fraction B was radioactive and contained no protein; it was identified as uridine by TLC. Fraction C was eluted at a gradient concentration of 0.2 M NH_4HCO_3. It contained 1100 nmol (ca. 2%) of modified RNase which incorporated 1070 nmol of [^{14}C]pUp and exhibited no nuclease activity. We refer to this fraction as the 1:1 adduct of RNase to pUp (RNase-pUp). Fractions D and E were identified as 5′-UMP and pUp, respectively, by TLC.

Gel filtration, on Sephadex G-50 superfine, of each of the protein fractions (A and C) revealed single peaks at the same elution volume as that of native RNase. This result was further confirmed by polyacrylamide gel electrophoresis where fractions A and C gave single bands which comigrated with native RNase. It should be noted that protein aggregates were not obtained under the reported experimental conditions.

Isolation and Identification of Cross-Linked Peptides. The labeled tryptic peptide Asn-67-Arg-85 was isolated from fraction C and was found to contain 1 mol of covalently bound radioactive inhibitor per mol of peptide. This peptide was partially digested with thermolysin. HVPE at pH 1.9 separated the resulting peptides into three radioactive and ninhydrin positive bands. Chromatography in the second dimension (Figure 2) separated these bands as follows: the slowest moving one revealed a single radioactive and ninhydrin positive band (A). The next band was separated into three ninhydrin positive bands (B, C, D) of which only B was radioactive. The third revealed two ninhydrin positive bands (E, F), only F being radioactive. The amino acid composition of peptides A-F and the ^{14}C content of the radioactive peptides are given in Table I.

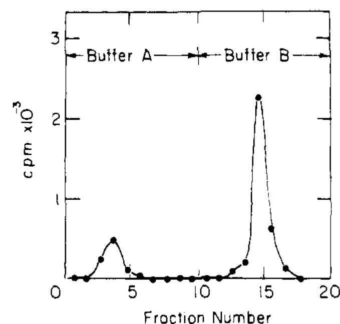

FIGURE 3: Separation of fragments containing amino acids and peptides bound covalently to [^{14}C]uridine by chromatography on DBAE-cellulose. Cross-linked tryptic peptide 67-85 was treated with alkaline phosphatase followed by digestion with Pronase and the mixture was applied to a column (6 × 0.9 cm) of DBAE-cellulose as described in the experimental procedure. Over 90% of the radioactivity was retained on the column in buffer A [0.05 M *N*-methylmorpholine (pH 7.7), 0.6 M KCl, 20% ethanol (v/v)] and was subsequently eluted with buffer B (0.05 M sodium acetate (pH 5.0), 0.2 M NaCl).

DBAE-Cellulose Affinity Chromatography of Fragments Containing [^{14}C]pUp. DBAE-cellulose has been used to purify RNA fragments having a free 3′-hydroxyl group by virtue of the capability of the dihydroxyboryl group incorporated in the resin to reversibly bind compounds with a *cis*-diol function (McCutchan et al., 1975). We have exploited this feature to separate fragments labeled with pUp from a proteolytic digest of the labeled peptide. The peptide was treated with alkaline phosphatase, in order to remove 3′-phosphate groups, prior to treatment with Pronase. The elution pattern of the Pronase digest (60 nmol) from the DBAE column is shown in Figure 3. Ninety percent of the radioactivity was retained on the column at pH 7.7 and was subsequently released at pH 5.5. The amino acid analysis of the retained fraction revealed the presence of Asp (30 nmol), Met-sulfone (43 nmol), and Ile (10 nmol). In a blank experiment, using a Pronase digest of nonmodified peptide 67-85, neither amino acids nor peptides were retained on the column.

Discussion

Our previous results indicated the Thr, Ser, Ile, and Arg residues of tryptic peptide 67-85 of RNase as suitable candidates for being modified by photochemical cross-linking to the pyrimidine ring of the enzyme's inhibitors. This speculation was based on the low values obtained for these residues in the amino acid analysis of the [^{14}C]pUp-labeled peptide 67-85 isolated from a tryptic digest of performic acid oxidized cross-linked RNase. However, the low specific activity of the modified peptide did not allow precise determination of the modified amino acids. It was necessary, therefore, to obtain a peptide containing one molecule of covalently bound inhibitor per peptide chain which will enable further analysis and identification of the modified residues. This was achieved by careful ion-exchange fractionation of the cross-linking reaction mixture which yielded a monomeric RNase molecule containing one covalently bound pUp residue (RNase-pUp). This modified RNase exhibited zero activity, as expected for a 100% modified enzyme with the inhibitor covalently bound at the active site. It should be noted that, under the irradiation conditions used in these experiments, protein-protein cross-linking did not take place, and destruction of light-sensitive amino

TABLE I: Amino Acid Composition of Peptides Obtained by Partial Thermolysin Digest of Tryptic Peptide Asn-67-Arg-85 of RNase Containing One Covalently Bound [^{14}C]pUp per Molecule of Enzyme.

Peptide	Amino acid composition																			[^{14}C]pUp (nmol) bound to peptide	Peptide [b] (nmol)	Spec radioact.
	67 Asn	Gly	Gln	70 Thr	Asn	Cys	Tyr	Gln	75 Ser	Tyr	Ser	Thr	Met	80 Ser	Ile	Thr	Asp	Cys	85 Arg			
A	3/3	1.04	1.8/2	2/3	3/3	1.8/2	1.5/2	1.8/2	2/2	1.5/2	2/3	2/3	0.9	2/3	0.75	2/3	3/3	1.8/2	0.9	30.4	32.3	0.94
B	2/2	1.04	2.4/2	0.84	2/2	0.9	1.5/2	2.4/2	2/2	1.5/2	2/2										25	
C												2/2	1	–	–	2/2	1	0.93	0.81	25.9	25.3	1.02
D	2/2	0.83	1.6/2	1.6/2	2/2	0.9	1.6/2	1.6/2	2/2	1.6/2	2/2	1.6/2	1.09	2/2							16	
E															1.1	0.5	1	1.1	0.98	17.4	34.9	0.5
F												0.83	1	1.18							22	

[a] Based on the theoretical number of aspartic acid residues (or methionine sulfone residue in peptide F), the theoretical number of residues which appear more than once in a peptide chain are given by the denominator. Cys and Met are in the oxidized form: cysteic acid and methionine sulfone, respectively. [b] The analysis was performed on 150 nmol of peptide Asn-67-Arg-85; the yield of peptides extracted from the paper after two-dimensional separation was ca. 50%.

acids was negligible. This could, most probably, be due to the increase (tenfold) in the reactant concentrations and their low conversion into products.

Peptide Asn-67-Arg-85 was the only radioactive tryptic peptide of RNase-pUp, and it contained one [^{14}C]pUp molecule per peptide chain. The peptide bonds between residues Ser-77-Thr-78 and Ser-80-Ile-81 are the most susceptible bonds to hydrolysis by thermolysin (Matsubara, 1970). Partial cleavage at these points should yield five peptides and these were indeed obtained, purified on paper, and their amino acid composition was determined (Figure 2 and Table I). Peptides B and D were identified as Asn-67-Ser-77 and Asn-67-Ser-80, respectively. Since they were not radioactive, the possibility that the photochemical cross-linking with pUp modified any residue in the sequence Asn-67 through Ser-77 can be excluded. Peptides C and E are the C-terminal complements of B and D, respectively, and are both radioactive. The cross-linking, thus, involved residues belonging to sequence 78–85.

The specific radioactivity of modified peptide 67–85 is unity. It contains, however, several modified amino acid residues. Modified peptide 67–85 comprises, therefore, several populations of peptides. Each of these populations contains a different modified residue. The cleavage pattern and the yields of the thermolytic peptides suggest that two such populations exist. The first one, in which Ser-80 and Ile-81 are modified, can be cleaved only between residues Ser-77 and Thr-78 to give peptides B and C. Peptide C contains 1 equiv of the radioactive inhibitor; however, it is deficient in two amino acids. This can be explained by assuming that primarily only one of the missing residues cross-linked with excited pUp; the second one lost its identity in a secondary reaction either with reactive intermediates (free radicals) or by cross-linking to the same molecule of pUp. This assumption is supported by the fact that radioactive peptides deficient either in Ser or Ile were not obtained. We further argue that Ser-80 is the initial target for the excited pUp, whereas its next neighbor Ile-81 is subsequently modified. This argument is based on the known reactivity of uracil derivatives in forming, under irradiation, addition products with alcohols which are analogous to the side chain of serine (Elad, 1976). The supporting evidence for this argument is provided by the DBAE-cellulose affinity chromatography experiment. Peptide 67–85 was exhaustively digested with alkaline phosphatase to remove the phosphate groups, followed by treatment with Pronase to break it into amino acids and peptides. The whole mixture was passed through a column which binds specifically compounds containing *cis*-diol moieties. Thus, amino acids and peptides which had been cross-linked to pUp were retarded by the column (Figure 3). The retarded fraction, after acid hydrolysis, yielded only Asp, Met-sulfone, and Ile in a 3:4:1 ratio. These amino acids must be the nearest neighbors of modified residues in peptides which could not be hydrolyzed by Pronase near a cross-linked residue. Thus, Asp and Ile are the nearest neighbors of Thr-82, whereas Met and Ile are the nearest neighbors of Ser-80. The fact that neither Ser nor Thr was found in this fraction indicates the absence of a peptide in which only Ile was modified.

In the second population of peptide 67–85, only Thr-82 was modified. Thus, the cleavage points of thermolysin remained intact. Four out of the five peptides expected from partial hydrolysis were isolated. The absence of peptide 78–85, in which only Thr-82 was modified, can be explained by a quantitiative cleavage at the Ser–Ile bond which is known to be the most sensitive to hydrolysis by thermolysin (Matsubara, 1970). Peptide E, the only radioactive peptide in this digest, contained half the radioactivity expected for a 1:1 addition product with pUp, while half of Thr-82 was missing. This result is difficult to account for without a detailed study of the photochemical mechanism of the cross-linking reactions and the chemical structure of the addition products. However, by analogy to the published work on the photochemical reactions of uracil derivatives with alcohols (Elad, 1976), it can be assumed that two types of addition products of threonine across the 5,6-double

bond of the pyrimidine ring were obtained: (I) C-C bond formation between C-6 of uracil and the β-carbon of the threonine (α to the hydroxyl); and (II) C-O bond formation between C-6 and the hydroxylic oxygen of the threonine. The latter ether type photoproduct is sensitive to acid and is hydrolyzed to give the unmodified starting materials. It can thus be speculated that Thr-82 in RNase underwent both types of modification. The portion of peptide E which underwent a type II modification lost its radioactivity during work-up with concomitant restoration of Thr-82. The type I modified peptide E is resistant to acid treatment and thus retained the modified Thr-82 residue.

We conclude, therefore, that the initial cross-linking of RNase with pUp involves mainly Ser-80 and Thr-82. Modification of Ile-81 depends on, and takes place subsequent to, that of Ser-80. These three residues are part of sequence 77-82 which forms the bottom of the binding site of RNase for the pyrimidine ring of nucleotides designated as site B_1 (Wyckoff, 1968; Richards and Wyckoff, 1971; Meadows et al., 1969). The specific modification of these consecutive residues means that the photochemical cross-linking occurs between a region on the protein which is in close association with the pyrimidine ring in the native structure of the enzyme-inhibitor complex.

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