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The Histidyl Residues in Ribonuclease-S

PHOTOOXIDATION IN SOLUTION AND IN SINGLE CRYSTALS; THE IODINATION OF HISTIDINE-12*

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SUMMARY

With molar ratios of methylene blue to ribonuclease-S of 1:30, histidyl residues *105* and *119* are destroyed by photooxidation at approximately equal rates. The loss of histidine *119* results in complete loss of enzymic activity; the loss of *105* is without effect on the activity.

Histidine-*12* in RNase-S is photooxidized at a slower rate than *105* or *119* and slower than the same residue in S-Peptide. Apparently histidine-*12* is partially protected in the RNase-S complex. Loss of histidine-*12* in S-Peptide results in loss of potential activity.

Photooxidation of S-Peptide causes the oxidation of methionine-*13* to the sulfoxide as well as the destruction of histidine-*12*. The sulfoxide can easily be reduced to yield a derivative where the only change is in histidine-*12*.

Monoiodination of histidine-*12* in S-Peptide on carbon-2(4) also resulted in complete loss of potential activity.

Estimates of ratio of S-Protein to S-Peptide derivative association constants relative to that for the unmodified peptide show reductions in affinity by factors of about 700 for methionine-*13* sulfone, 1900 for photooxidized histidine-*12*, 3200 for 2(4)-iodohistidine-*12*.

The loss of histidine-*119* markedly lowers the affinity of S-Protein for native S-Peptide.

The photooxidation reaction was carried out in the solid state on single crystals of RNase-S. The qualitative effects were identical with those seen in solution, although the overall rate was slower.

A comparison of x-ray diffraction patterns of native and photooxidized crystals of RNase-S showed only slight intensity changes. There was no evidence of any disordering of the crystal lattice. It is tentatively concluded that only small general conformational changes can have occurred, and that the activity effects can probably be attributed directly to the loss of the imidazole residues.

A study of the methylene blue-catalyzed photooxidation of bovine pancreatic ribonuclease was reported some years ago by Weil and Seibles (1). Their data indicated that the loss of enzymic activity was accompanied by the loss of 1, or perhaps 2, residues of histidine with little or no change in any of the other amino acids. Extensive photooxidation resulted in the loss of 3 of the 4 histidyl residues. Subsequent studies on inactivation of the enzyme with haloacetate ions by Barnard and Stein (2) and by Gundlach, Stein, and Moore (3) also pointed to the importance of histidyl residues. This work and the later detailed investigations of Crestfield, Stein, and Moore (4) have identified histidine-**119** and histidine-**12** as 2 residues closely associated with the catalytic properties of the intact protein. An excellent summary has been given by Hummel and Kalnitsky (5). More recent work by Heinrikson *et al.* (6) on the reactivity of the protein with a variety of halo acids has confirmed the importance of residues **12** and **119**, and, in addition, has provided an extensive set of changes in absolute and relative reactivity for these two groups depending on the structure of the halo acid used. Convincing proof of the interrelation between these 2 residues comes from the reports by Fruchter and Crestfield (7) on the alkylation of ribonuclease dimers.

Investigations on the modified enzyme RNase-S[1] have confirmed many of the conclusions drawn in the studies referred to above. It was shown by Richards (8) that both the peptide and protein components of RNase-S could be separately inactivated by photooxidation, but the products were not further characterized. The synthetic work of Hofmann and his colleagues (summarized by Finn and Hofmann (9)) shows clearly that no activity in a protein-peptide complex can be demonstrated unless the residue equivalent to histidine-**12** is included in the synthetic peptide.

The present study was undertaken to identify the residues affected by photooxidation and to compare the modification of the enzyme by this reaction in solution and in the crystalline state. Observations on the latter point are possible because methylene blue is soluble in saturated ammonium sulfate and only very small amounts of this catalyst are required to effect the oxidation.

A series of experiments on a different problem has led to the specific iodination of histidine-**12** in S-Peptide. Some results of this work are included for their relevance to the general problem of the function of the imidazole groups in this system.

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[1] The abbreviations used are: RNase-S, ribonuclease-S, the derivative obtained from the native enzyme by enzymatic cleavage of a single bond; S-Peptide, the peptide component of RNase-S; S-Protein, the protein component of RNase-S $MetO_2$-S-Peptide, a derivative of S-peptide where the single methionyl residue has been oxidized to the sulfone.

EXPERIMENTAL PROCEDURE

Materials

Native crystalline bovine pancreatic ribonuclease was obtained from L. Light and Company, Ltd. RNase-S, S-Protein, and S-Peptide were prepared as described by Gordillo, Vithayathil, and Richards (10). RNase-S was crystallized from ammonium sulfate by the method of Doscher and Richards (11) or by modifications introduced by Wyckoff.[2] Methylene blue was obtained from the National Aniline Division, Allied Chemical and Dye Corporation. Trypsin was purchased from Sigma and chymotrypsin from Calbiochem. Sephadex was the product of Pharmacia. Other resins were obtained either from Calbiochem or the Fisher Scientific Company. All other reagents and solvents were commercial analytical grade products unless otherwise indicated.

A sample of 2(4)-iodohistidine was synthesized by the method of Brunings (12). Elementary analyses were performed by Galbraith Laboratories, Inc.

Methods

Enzymic Activity—All measurements of RNase activity were made with cytidine 2′,3′-phosphate (Sigma), by means of the spectrophotometric method of Crook, Mathias, and Rabin (13). The assays were conducted at 25° in 0.1 M Tris-0.3 M NaCl buffer, pH 7.0, with a substrate concentration of 2.77×10^{-4} M and enzyme concentration in the range 5 to 30 μg per ml. The hydrolysis of the substrate was followed by measurement of the increase in absorbance at 284 mμ. When the concentration of the enzyme exceeded 15 μg per ml, the initial velocity was measured as described by Crook *et al.* (13). For enzyme concentrations lower than 15 μg per ml, the initial activity was taken as the slope of the curve of change in absorbance with time during the first 3 min.

Photooxidation of S-Peptide—A 5-ml portion of a 0.1% solution of S-Peptide in 0.2 M NH_4HCO_3, pH 8.5, was irradiated at room temperature in the presence of 0.001% methylene blue (molar ratio of peptide to methylene blue, about 18:1) in a 50-ml beaker. Illumination was provided by a 100-watt tungsten bulb positioned 5 inches above the surface of the solution. During irradiation the solution was continuously stirred with a magnetic stirrer. After irradiation the solution was freed of methylene blue by running it over a column (34 × 2.4 cm) of Sephadex G-25, medium grade, with 0.1 M NH_4HCO_3, pH 8.5, as the eluent. The ammonium bicarbonate was removed by lyophilization.

Photooxidation of RNase-S in Solution—The procedure was essentially the same as for S-peptide except that a 1% solution of RNase S was employed and the molar ratio of protein to methylene blue was 30:1. The methylene blue was removed on a Sephadex column as above. When the photooxidized RNase was chromatographed on an Amberlite CG-50 column, there was no necessity for a separate operation to remove the methylene blue, which was adsorbed by the carboxylic acid resin.

Photooxidation of Crystals of RNase-S—RNase-S crystals (the size of the crystals depending on the experiment) were suspended in 0.2 ml of 85% saturated $(NH_4)_2SO_4$ adjusted to pH 7.7. The solution of $(NH_4)_2SO_4$ contained enough methylene blue to give a molar ratio of protein to dye of approximately 30:1. The suspension was placed in the hollow of a depression slide. The suspension and a trapped air bubble were covered with a cove slip. The crystals were allowed to equilibrate with the meth ylene blue solution for 48 hours in the dark. At the end of th period the depression slide was fastened to the top of a magneti stirrer and the crystal suspension therein was irradiated as de scribed for the solution studies. The stirring of the crystals i the methylene blue solution was accomplished by turning th motor of the magnetic stirrer to full speed. The vibrations pro vided by the running motor caused enough stirring of the crysta in the medium to allow for oxygen exchange without damage t the crystals. The magnetic stirrer was cooled with a fan to pre vent heating of the crystals during the several hours required fo photooxidation.

Preparation of Iodinated S-Peptide—S-Peptide (5 mg) was dis solved in 2 ml of 0.3 M glycine-0.025 M NaCl buffer, pH 8.5, at 0° A cold dilute solution (0.25 ml) of iodine monochloride, prepared according to Koshland *et al.* (14), containing 5.5 μmoles of I_2 was then added. The solution was allowed to stand for 1 min and was then put immediately on a column (34 × 2.4 cm) of Sepha dex G-25, medium grade, to remove the salt. The elution wa carried out with 0.3 M NH_4HCO_3 buffer, pH 8.5, which could subsequently be removed by lyophilization. By this procedure about 60% of the peptide mixture was obtained free of buffe salts. The remaining 40% was lyophilized, dissolved, and recycled. The salt-free peptide mixture was then subjected to electrophoresis as described in the legend to Fig. 5.

Amino Acid Analyses—Protein and peptide samples for amin acid analysis were hydrolyzed in 6 N hydrochloric acid in sealed evacuated ampules at 110° for 24 hours. The amino acid mix tures were analyzed on automatic equipment as described by Moore and Spackman *et al.* (15, 16) except that the "long" col umn was 50 cm and the short column was 14 cm, with flow rates of about 75 and 66 ml per hour, respectively. The buffer change on the "long" column was made 140 min after the start of the run. The last amino acid normally observed was phenylalanine appearing at 310 to 315 min (or 340 to 346 ml). In the experi ments with iodohistidine, the buffer was changed again at 25 min to that normally used for the short column (*i.e.* pH 5.28, 0. M). The iodohistidine peak then appeared at 435 min well sep arated from phenylalanine. No satisfactory separation of iodo histidine from tyrosine and phenylalanine was obtained on th small column runs. In the photooxidized samples of RNase-S small amounts of two unknown components were observed a 365 and 395 min in this same system.

Treatment of a reference sample of monoiodohistidine unde the usual conditions for protein hydrolysis indicated less than 10% loss. The small amount of lost derivative appeared as fre histidine in the hydrolysate.

Peptide Maps—Peptide maps were prepared on Whatman N 3MM paper. Chromatography in the first dimension was ca ried out with 1-butanol:acetic acid:water, 4:1:5, as solvent b the descending technique. In the second dimension ionophoresi by the cooled plate method was used with pyridine:acetic acid water buffer, 1:10:89, pH 3.6, at 16 volts per cm for 5 hours. I some experiments the electrophoresis was performed with Savant high voltage paper electrophoresis tank and power suppl The voltage across the paper was 40 volts per cm and the curren varied between 120 and 180 ma. Savant EC-123 was used a the coolant. The dried chromatograms were developed wit either 0.25% ninhydrin (unless otherwise specified) or Pauly re agent spray (17). The Pauly reagent was mainly used for th

[2] H. W. Wyckoff, personal communication.

identification of histidine-containing peptides and also to identify those peptides which did not show up with the ninhydrin reagent.

The peptide mixtures were prepared as follows: 20 mg of native or photooxidized RNase-S was oxidized with performic acid as described by Hirs (18). A 2% solution of this performic acid-oxidized enzyme was made in 0.2 M NH_4HCO_3 buffer, pH 8.5, and a sufficient volume of a 1% solution of trypsin in water was added to give a ratio of trypsin to RNase of 1:100. The digestion was carried out for 3 hours at 37°. Then the same quantity of a 1% solution of chymotrypsin was added and the digestion continued for another 3 hours at 37°. The digested mixture was adjusted to pH 2.5 with 3 N HCl. Between 50 and 100 μl of this mixture (equivalent to 1 to 2 mg of enzyme) were spotted directly on the paper, and the buffer salt was volatilized in a stream of air.

Chromatography on the carboxylic acid resin Amberlite IRC-50 (CG-50), type 2, was performed as described by Hirs, Moore, and Stein (19) with 0.2 M phosphate buffer, pH 6.1, as the eluent.

X-ray Studies—Single crystal x-ray diffraction patterns were recorded on a Supper precession camera with copper $K\alpha$ radiation from a General Electric CA-7 sealed tube and XRD-5 power supply. The crystals were mounted in thin walled, sealed, glass capillaries in vapor equilibrium with the mother liquor (20).

RESULTS

The activity loss during photooxidation of RNase-S in solution is shown in Fig. 1*a*. The activities are expressed as percentage of that observed before the illumination of the solution began. Three aliquots were removed at each time. The first was assayed directly. The second was assayed in the presence of added S-Peptide, and third with added S-Protein. The last two assays were used to test for the presence of activatable S-Protein and S-Peptide, respectively, in the photooxidized sample. Similar data on a crystal preparation are shown in Fig. 1*b*. In this case the portions of crystals removed from the photooxidizing suspensions varied slightly in weight of protein. These samples were sedimented, dissolved in water, and the protein concentration estimated from measurements of absorbance at 280 mμ. The solutions were adjusted to the same protein concentration and aliquots then used for the assay series as described above.

In the solution studies the liquid was stirred vigorously so that saturation with air was maintained, and all parts of the solution came close to the interface near the source of illumination (21). The strong absorption of these solutions in the visible region due to the high concentration of methylene blue (0.01%) thus posed no problem. With the crystals, however, it was noted that the methylene blue was taken up preferentially into the crystal lattice. With the amounts of dye used for solution studies, the crystals were stained an intense blue. The very high absorbance prevented visible light from reaching the center of the crystals and the oxidation process became very nonuniform. Actually very little activity loss was observed even over quite long periods of time, presumably due to this extinction effect. When the amount of methylene blue was markedly reduced, to the range of 1 methylene blue molecule for every 20 or 30 protein molecules, the crystals appeared just barely blue to the eye. The absorbance of crystals of even 0.5 mm thickness was undoubtedly less than 0.5 in the 500 mμ region. Although slow because of the inherent dependence on dye concentration, the oxidation reaction now proceeded well.

The diffusion of oxygen into the crystals might also, in principle, be a limiting factor. However, the crystals were maintained close to the air-liquid interface with constant gentle agitation. One attempt at the use of pure oxygen instead of air did not markedly affect the rate of activity loss. The process appears to be limited by the concentration of photoactivated methylene blue. Because of the low molar ratios employed, the dye molecules were behaving as true catalysts and each had to affect a respectable volume of the lattice during the course of the reaction.

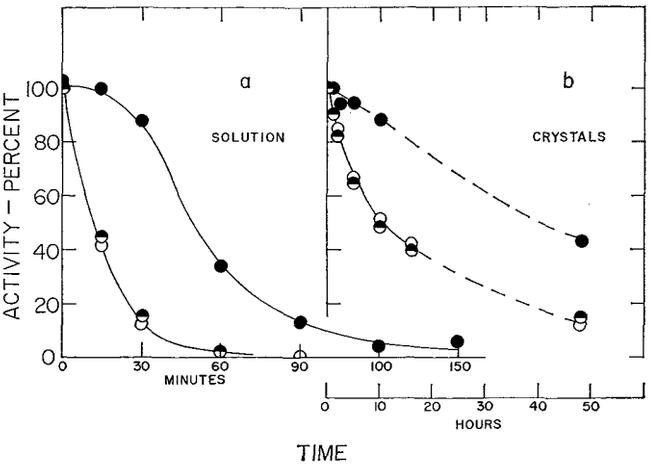

FIG. 1. Activity loss on photooxidation of RNase-S. In all assays, cytidine 2′,3′-phosphate was used as substrate. ○, direct assay of aliquots from reaction mixture; ◓, assay of aliquots containing added S-Peptide; ●, assay of aliquots containing added S-Protein. Samples of crystal suspensions were dissolved and adjusted to equivalent protein concentrations before assay. The molar ratio of methylene blue to protein in solution was 1:30, and in the crystals was approximately 1:30.

Amino acid analyses of the samples of RNase-S which were inactivated to the extent of 74% in solution and 77% as crystals are given in Table I. In repeated analyses the only large differences from the controls appeared in the histidine content. (Factors for precise quantitation of these analyses are uncertain. At least some of the "lost" histidine in the photooxidation of small peptides appears as ninhydrin-positive material in many places on the chromatogram, frequently under the usual amino acid peaks.[3]) Tyrosine clearly was unchanged in comparison with the control. (The measured number for tyrosine has been consistently low compared to the expected value, even in the control. At least part of the discrepancy is caused by the formation of chlorotyrosine during performic acid oxidation (18).) Photooxidation may convert methionine to the sulfoxide. Such an effect would not have been observed in these analyses, as the samples were further oxidized with performic acid and the methionine was measured as the sulfone. This effect presumably was observed in experiments with S-Peptide, as discussed below, but no direct analyses for sulfoxide content have been carried out.

Comparison of a number of peptide maps of digests of unmodified and photooxidized RNase-S have consistently shown the disappearance of two major spots, both Pauly-positive, in the map of the unmodified protein (Fig. 2). Four very weak ninhydrin-positive spots appeared on most of the maps made from photooxidized material. For the purpose of identifying the peptides represented by *Spots 1* and *2* in the amino acid sequence,

[3] K. Stiller and H. Wasserman, personal communication.

TABLE I

Amino acid analyses of derivatives of RNase-S and S-Peptide

All values are uncorrected for losses on hydrolysis. In calculating molar ratios, the sum of the values for glutamic acid, alanine, valine, and phenylalanine was taken to be 36 for the RNase-S analyses.

Amino acid	Residues/molecule							
	RNase-S[a]				S-Peptide[b]			
	Theory (22)	Native	Solution, photo-oxidized	Crystal, photo-oxidized	Theory (23)	Native	Photooxidized	Iodinated
Cysteic acid	8	7.2	7.3	7.6				
Methionine sulfoxides							0.2	
Aspartic acid	15	14.3	14.5	15.3	1	1.0	1.0	0.9
Methionine sulfone	4	3.8	3.8	3.8				
Threonine	10	8.9	9.1	8.8	2	1.9	1.9	1.9
Serine	15	13.2	14.1	14.5	3	2.8	2.5	2.6
Glutamic acid	12	11.3	11.5	11.2	3	3.0	3.1	3.3
Proline	4	3.7	3.7	3.8				
Glycine	3	3.3	3.0	3.5				
Alanine	12	12.8	12.3	12.5	5	4.4	4.2	4.3
Valine	9	9.2	9.2	9.2				
Methionine					1	0.9	0.7	0.8
Isoleucine	3	2.3	2.4	2.6				
Leucine	2	1.8	2.2	2.2				
Tyrosine	6	4.9	5.1	5.2				
Phenylalanine	3	2.7	3.0	3.2	1	1.0	1.0	1.0
Unknown 1[c]			0.3	0.4				
Unknown 2[c]			0.3	0.2				
Iodohistidine[c]								0.8
Lysine	10	9.7	9.7	10.1	2	2.1	2.0	2.1
Histidine	4	**3.6**	**1.9**	**1.9**	1	**1.0**	**0**	**0.1**
Arginine	4	3.7	3.8	3.4	1	0.9	0.9	1.1

[a] Samples were oxidized with performic acid before hydrolysis.

[b] Klee (24) has shown that elastase produces a 19-residue peptide missing the COOH-terminal alanine of S-Peptide. Doscher has informed us (personal communication) that several bacterial proteinase preparations produce peptide mixtures containing at least two species, 20- and 21-residue peptides. These observations provide a possible rationale for the low value for alanine found in this particular "S-Peptide" preparation.

[c] Peaks observed on "long" column after phenylalanine (315 min); see "Methods." Unknown 1, 365 min; Unknown 2, 395 min; iodohistidine, 434 min. Quantities of Unknowns 1 and 2 are based on color value for phenylalanine.

the peptide map was developed with 0.025% ninhydrin solution, instead of the usual 0.25%. The spots were cut out, washed with acetone to remove excess ninhydrin, eluted with 0.1 N HCl, and then hydrolyzed for 21 hours and analyzed. The compositions are shown in Table II. The only sequences from the native enzyme which would be expected to occur in a tryptic-chymotryptic digest, and which approximate the measured values, are also listed in Table II (22). There can be little doubt that the two histidyl residues principally affected by the photooxidation reaction are those in positions **105** and **119**. Similar maps of material photooxidized in the crystalline state gave identical results and thus are not shown separately.

Enzyme samples were chromatographed on the weak cation exchange agent, Amberlite IRC-50. The native enzyme is substantially retarded on this resin, as shown in Fig. 3*a*. The enzyme activity measured in aliquots of the column fractions accurately follows the optical density readings at 280 mμ. Material completely inactivated by photooxidation moved at, or very close to, the column front (Fig. 3*b*). A partially inactive sample showed four peaks in this same system (Fig. 3*c*). The first corresponded to fully inactive material and the last to presumably unaltered enzyme. Between these two were two new peaks of roughly the same area, the first apparently as active as the starting material and showing a loss of only 1 histidine residue on amino acid analysis, and the second completely inactive. The simplest explanation is that residues **119** and **105** are independent and are photooxidized at nearly the same rate. One of the monodegraded derivatives is fully active and the second, inactive. In confirmation, material recovered from the active peak showed the loss of *Spot 1* while the Pauly-positive *Spot 2* was still evident. The loss of histidine-**105** apparently does not does not affect the activity of the enzyme.

Because the totally inactive material runs close to the column front, as does S-Peptide itself (23), no firm conclusions are warranted, on the basis of these data, as to whether the peptide binds to the doubly modified S-Protein moiety or not. However, assay of column fractions in the presence of added S-Protein gave an activity peak in the leading edge of the optical density peak. The height of this peak was less than that expected from the peptide equivalent to the optical density peak. It thus appears that some separation of native peptide from inactivated S-Protein did occur on this resin, and that part of the peptide was inactivated also. In passing, it may be noted that the modified S-Protein appears near the column front and not strongly adsorbed to the resin as is the case with unmodified S-Protein.

A column (34 × 2.4 cm) of Sephadex G-25, medium grade,

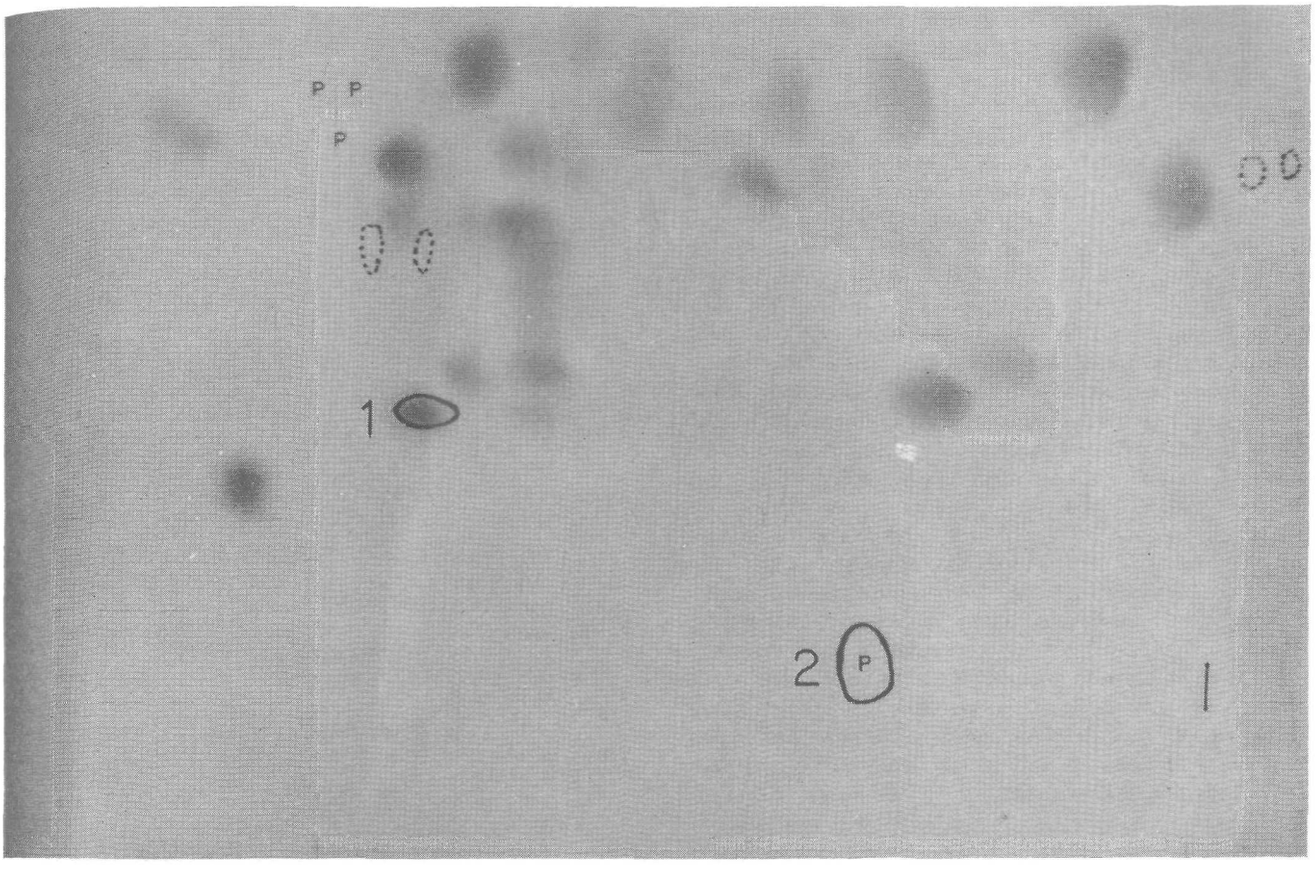

FIG. 2. Peptide maps of tryptic-chymotryptic digests of RNase-S and photooxidized RNase-S. The photograph shows the ninhydrin-positive spots from a digest of the native enzyme. The symbol *P* indicates the location of ninhydrin-negative areas which gave a positive Pauly reaction. (Other Pauly-positive spots are not shown.) The equivalent map from a photooxidized sample of the enzyme was identical with that shown except that the spots encircled with *solid lines* (*Spots 1* and *2*) disappeared, and very faint ninhydrin-positive spots appeared at the areas indicated by the *broken lines*. No new Pauly-positive spots were observed. Origin is at *upper left;* chromatography in *vertical direction*, 1-butanol:acetic acid:water, 4:1:5; electrophoresis in *horizontal direction* cathode at right, pyridine-acetic acid buffer, pH 3.6.

with 0.2 M NH_4HCO_3, pH 8.5, as the eluting liquid, easily distinguished between RNase-S and S-Peptide when run as separate samples (Fig. 4). The peptide was detected by assay in the presence of added S-Protein. When a photooxidized sample of RNase-S was run over the same column, the small amount of residual activity in this material ran at the same place as the RNase-S peak. When extra S-Protein was added to a second set of aliquots from this run, additional activity appeared in the same position. No activity was found in the position corresponding to free S-Peptide. The recovered activity was not equivalent to that from the same amount of RNase-S. Thus some oxidation of the peptide component had occurred in the photooxidized sample. However, the unaffected S-Peptide clearly had not dissociated from the altered protein component.

Studies on the photooxidation of free S-Peptide in solution showed that activity loss occurred at about the same rate as shown for RNase-S in Fig. 1. The peptide is oxidized much more slowly when in a complex with S-Protein, thus histidine-**12** must be substantially protected from this type of reaction in RNase-S.

Ionograms of S-Peptide and various derivatives are shown in Fig. 5. The particular preparation used in this series of experiments contained a small amount of ninhydrin-positive material as a contaminant, shown as the *broken line spot* in Fig. 5*a*. The

TABLE II

Identification of peptides which disappear on photooxidation of RNase-S

Amino acid analyses of spots eluted from peptide maps equivalent to that shown in Fig. 2. All amino acids not listed were absent or present only in traces.

Amino acid	Spot 1		Spot 2	
	Found[a]	Calculated for residues **105–115**	Found[b]	Calculated for residues **116–120**
Cysteic acid	0.9	1		
Aspartic acid	1.0	1		
Glutamic acid	0.9	1		
Proline	0.9	1	1.0	1
Glycine	1.0	1		
Alanine	1.0	1		
Valine	1.0	1	1.6	2
Isoleucine	1.5	2		
Tyrosine	0.8	1		
Phenylalanine			1.0	1
Histidine	0.8	1	0.9	1

[a] Molar ratios based on alanine = 1.0.

[b] Molar ratios based on phenylalanine = 1.0.

photooxidized sample (Fig. 5*b*) showed only one principal spot, which was used for amino acid analysis. There was no evidence of any unmodified peptide or of any fragments due to cleaving of the chain. The contaminant appeared again in about the same position. The iodinated peptide (Fig. 5*c*) gave a series of spots. The less intense spots, *1*, *2*, and *4*, gave inconclusive amino acid analyses. They did not approximate the composition of the whole peptide and presumably represent cleavage or degradation products and may be mixtures of peptides. The principal spot, *3*, is the iodopeptide, the analysis of which is given in Table I. The photooxidized peptide and material equivalent to *Spot 3*, eluted from preparative ionograms, were used in the competition assays described below.

Fully photooxidized peptide or purified iodopeptide showed only very slight activity even at peptide to protein ratios of 100 or more. Since evidence to be discussed below indicated that complex formation with S-Protein had proceeded to the extent of at least 70 to 80% under these conditions, it is concluded that such complexes have less than 2 or 3% the activity of RNase-S. Any more definitive statement on the inherent activity of these peptide derivatives is not warranted because of the possibility of contamination of these samples with unchanged S-Peptide.

Since activity measurements could not be used directly to demonstrate interaction between S-Protein and these peptide derivatives, estimates of binding were made by inhibition studies. S-Peptide itself is too tightly bound to be useful for this purpose. Performic acid oxidation simply and quantitatively produces $MetO_2$-S-Peptide, a derivative where the single methionine

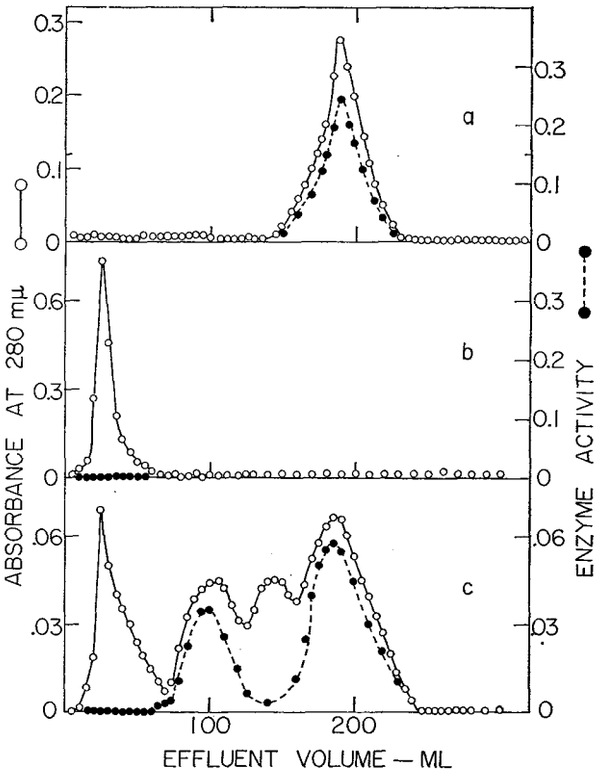

FIG. 3. Chromatograms of photooxidized RNase-S on an Amberlite IRC-50 (CG-50 Type II) column (30 × 1.1 cm). The eluting buffer was 0.2 M potassium phosphate, pH 6.10. *a*, 15 mg of fully active RNase-S; *b*, 15 mg of enzyme after total inactivation by photooxidation; *c*, 15 mg of enzyme after 40% activity loss through photooxidation.

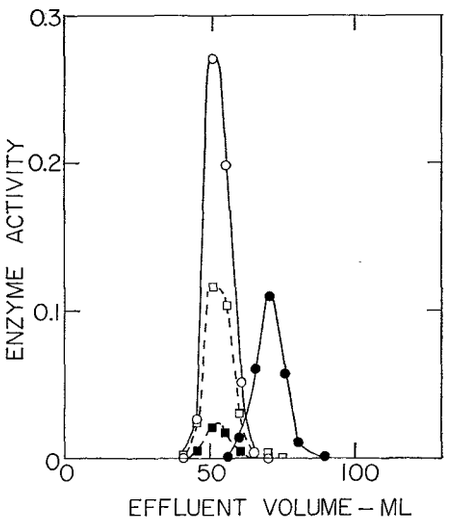

FIG. 4. Chromatograms on a Sephadex G-25 column (34 × 2.4 cm), medium grade. ○, RNase-S; ●, S-Peptide, aliquots of column fractions assayed in the presence of added S-Protein; ■, photooxidized sample of RNase-S; □, additional aliquots from previous run assayed in presence of added S-Protein.

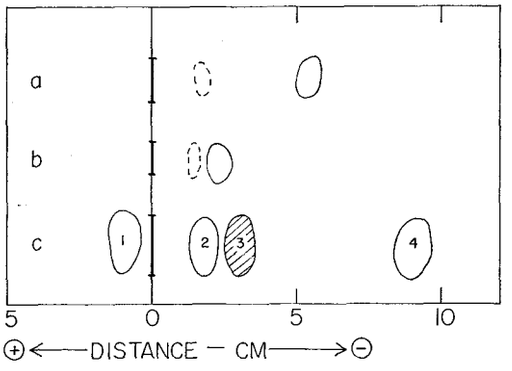

FIG. 5. Ionograms of native, photooxidized, and iodinated S-Peptide in pyridine-acetate buffer, pH 5.7. The ionophoresis was carried out for 5 hours at 16 volts per cm on a Whatman No. 1 paper, using a cooled plate apparatus; *a*, untreated S-Peptide. 100 μg; *b*, photooxidized S-Peptide, 100 μg; *c*, iodinated S-Peptide. 500 μg. The major spot is *shaded*. A ninhydrin spray was used in developing the spots.

residue has been oxidized to the sulfone (25). This material is much more weakly bound to S-Protein, and competition with inactive derivatives is easily measured under the ordinary assay conditions. The concentration of active S-Protein:$MetO_2$-S-Peptide complex is measured by the assay. From previous work this complex is known to have 90 to 100% of the activity of RNase-S (25). The assays are carried out at a substrate concentration well below the Michaelis constant and thus the protein-peptide equilibria are not sensibly disturbed. (There is no evidence for tight binding of substrate to areas other than the active site which might thus affect the equilibria without being detected by the assay techniques.)

The equations used in the treatment of the data are as follows:

$$P + p_a \rightleftharpoons^{K_a} E_a \quad (1)$$

$$P + p_i \rightleftharpoons^{K_i} E_i \quad (2)$$

Define:

$(P)_T = (P) + (E_a) + (E_i)$ = total concentration of S-Protein
$a = (E_a)/(P)_T$ = measured activity as fraction of the maximum possible activity
$i = (E_i)/(P)_T$
$(p_a)_T = (p_a) + (E_a)$ = total concentration of active peptide
$(p_i)_T = (p_i) + (E_i)$ = total concentration of inhibiting peptide
$r_a = (p_a)_T/(P)_T$
$r_i = (p_i)_T/(P)_T$

$$K_a = (E_a)/(P)\ (p_a) \quad (3)$$

$$K_i = (E_i)/(P)\ (p_i) \quad (4)$$

Eliminate (P) from Equations 3 and 4, put in definitions, and rearrange to get:

$$i = r_i \Big/ \left[1 + \frac{K_a}{K_i}\left(\frac{r_a - a}{a}\right)\right] \quad (5)$$

Substitute Equation 5 and definitions into Equation 3 and solve for K_i:

$$K_i = \left[\frac{K_a(1 - a)(r_a - a)}{a} - \frac{1}{(P)_T}\right] \Big/ \left[r_i - 1 + a - \left(\frac{a}{r_a - a}\right)\frac{1}{K_a(P)_T}\right] \quad (6)$$

TABLE III

Assays showing competition by inactive peptide derivatives

Substrate cytidine 2′,3′-phosphate 2.77×10^{-4} M in 0.1 M Tris-0.3 M NaCl, pH 7.0, buffer, 25°; S-Protein, 25 μg per ml (2.2×10^{-6} M); $MetO_2$-S-peptide, 4.7 μg per ml ($r_a = 1$) to 28.8 μg per ml ($r_a = 6$). Maximum activity for this amount of S-Protein when fully combined with S-Peptide was 0.043 ΔA_{284}/min. The fractional activities were calculated with this number.

Peptide inhibitor	Molar ratio, $MetO_2$-S-Peptide to S-Protein, r_a	Measured activity (initial ΔA_{284}/min $\times 10^3$)	Fractional activity, a	Equation 7[a]: $K_a(M^{-1}) \times 10^{-5}$	Equation 6: $K_i(M^{-1}) \times 10^{-4}$
None	1	9	0.21	1.5	
	1.5	12	0.28	1.5	
	2	14	0.33	1.3	
	3	19	0.44	1.4	
	4	23	0.54	1.5	
	5	27	0.63	1.8	
	6	29	0.68	1.8	
				1.5[b]	
Photooxidized peptide					
100 μg, $r_i = 21$	1	8	0.19		0.4
	2	14	0.32		0.3
	3	17	0.40		0.7
	4	20	0.46		0.8
	5	22	0.51		1.0
	6	25	0.58		0.7
					0.6[b]
Photooxidized-reduced peptide					
100 μg, $r_i = 21$	1	4	0.09		4.5
	2	7	0.16		5.0
	3	9	0.21		5.8
	4	11	0.26		6.0
	5	15	0.35		4.3
	6	17	0.40		4.2
25 μg, $r_i = 5.3$	5	21	0.49		6.0
250 μg, $r_i = 53$	5	6	0.14		6.9
					5.3[b]
Iodinated peptide					
100 μg, $r_i = 21$	1	6	0.14		1.8
	2	8	0.19		3.7
	3	10	0.23		4.7
	4	15	0.35		2.9
	5	16	0.37		3.6
	6	21	0.49		2.1
					3.1[b]

[a] Binding studies not reported in this paper (Kenkare, unpublished) provide an estimate for the association constant of native S-Peptide under the same conditions of about 1×10^8 M^{-1}.

[b] Averages.

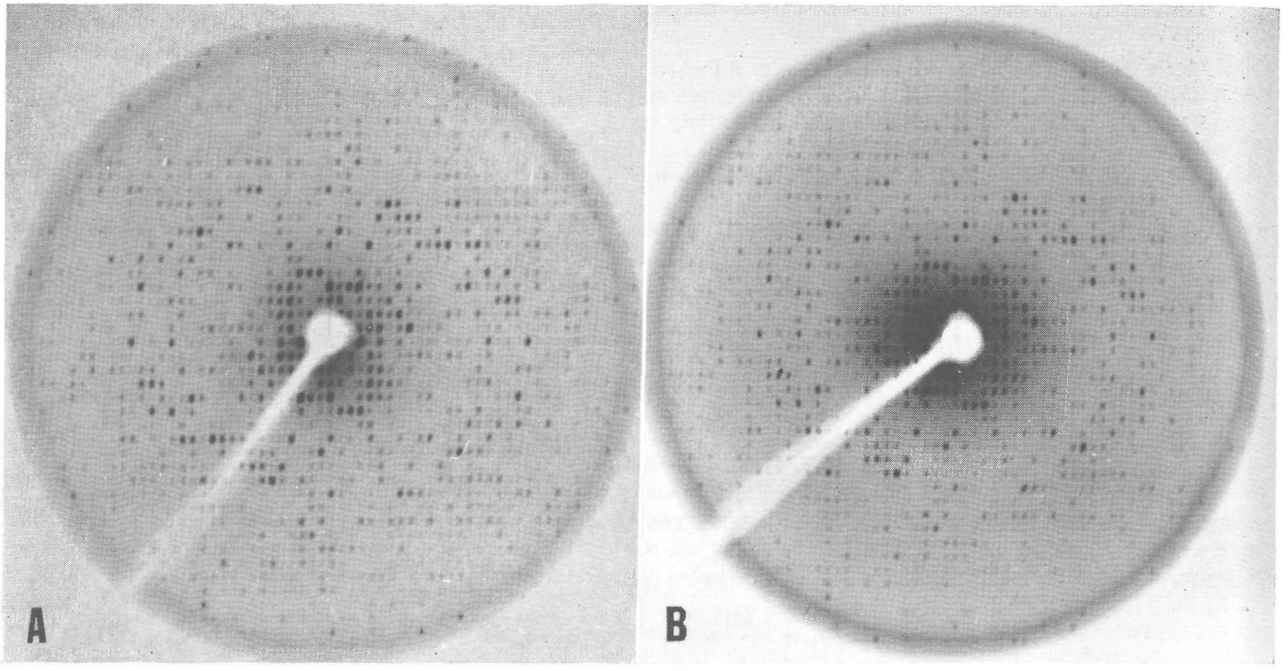

FIG. 6. Single crystal precession photographs. *A*, untreated RNase-S; *B*, RNase-S after 90% inactivation by photooxidation in the presence of methylene blue in the crystalline state. The precession angle was 20°; thus the outermost spots correspond to spacings of about 2.5 A. Both crystals came initially from the same batch of the Z-form of the enzyme. The (hol) plane of the monoclinic cell is shown with the reciprocal a-axis vertical and the reciprocal c-axis horizontal.

In the absence of any inhibitory peptide, Equation 3 yields:

$$K_a = \frac{a}{(P)_T(1 - a)(r_a - a)} \quad (7)$$

The assay data are shown in Table III along with the derived values of the association constants.

Although the amino acid analyses of the photooxidized peptide did not show any detectable methionine sulfone, the possibility of oxidation to the sulfoxide could not be ruled out. A sample of the photooxidized peptide (2.5 mg in 2.5 ml) was treated with 25 mg of sodium thioglycolate at pH 3.0 for 64 hours at 45–50° to reduce any sulfoxide that may have formed. Control studies with known samples of methionine sulfoxide indicated that these conditions should be more than sufficient to effect complete reduction to methionine. The binding studies with this sample are also shown in Table III. The increase in binding constant of almost a factor of 10 is presumptive evidence that the photooxidized peptide contained at least a substantial fraction of the methionine in the oxidized form.

Single crystal precession patterns were obtained on a number of different preparations which had been inactivated to varying degrees by photooxidation in the crystalline state (Fig. 6). No detailed interpretation of these patterns has been undertaken so far. However, a number of useful qualitative conclusions can be drawn. None of the patterns showed any significant change in unit cell dimensions. The general intensity distribution was very similar in all cases. In particular, there was no visual evidence for any marked change in the effective Debye temperature factor. Any substantial disordering of the lattice would result in a marked increase in this factor which could easily be seen by an increase in the fall off of the average intensity at the higher Bragg angles. Careful comparison of the original photographs does show small intensity changes in many reflections as must, of course, be the case. The destruction of the imidazole rings of 2 histidine residues causes the redistribution of a number of atoms, and the resulting changes in electron density at these sites will be reflected in the diffraction pattern. The maximum change in conformation that could occur within the limits imposed by the observed average change in intensity of the various reflections is not yet known. Crick and Magdoff (26) have estimated the effects of certain types of structural change on the diffraction pattern of a protein crystal. Even if they have overestimated the sensitivity of the pattern to such changes, the magnitude of the conformation change in the present instance is probably not large.[4]

In this type of reaction where the penetration of reagent (O_2) and light into the crystal might both be problems, it is conceivable that the crystal might become markedly disordered in the surface layers and retain the order of a true crystal only in a small unaltered central region. The effect on the diffraction pattern would be a decrease in absolute intensity of all the crystalline reflections and an increase in the background due to amorphous scattering and no change in relative intensities. Absolute intensity measurements are difficult because of the differing crystal mounts and the uncertain absorption corrections. A very crude attempt was made in this direction by drying and weighing the actual crystals used after the diffraction patterns

[4] A referee of this paper, to whom we are indebted, has pointed out that the intensity changes appear to be similar in magnitude to those produced by heavy atom isomorphous derivatives in other proteins. Since no large localized change in electron density of that sort can occur in the present instance, substantial shifts in a number of the lighter atoms comprising the protein itself may be indicated. This problem cannot be solved at the moment in the absence of more definitive x-ray studies.

had been collected. The intensities based on the crystal weight (they were all very similar in size) certainly did not vary by more than a factor of 2 or 3. Since well over 90% inactivation was obtained in some of the preparations, it appears likely that the diffraction patterns do, in fact, reflect the structure of the altered protein in the lattice and not the residue of unreacted material.

DISCUSSION

The activity loss attending the destruction of the ring of either histidine-**12** or histidine-**119** confirms the conclusion, already drawn from studies of the alkylation of these same residues, that both are "essential" for catalytic activity. The single crystal results and the attendant diffraction patterns would seem to rule out any extensive conformational change. However, a detailed interpretation of the intensity changes that are observed is required for a more definitive statement. Thus an active role for these histidyl residues at the catalytic center is still the most probable explanation of all the available data.

A structural change of some sort, albeit perhaps very small, is implied by the binding data. The modification of histidine-**12** or histidine-**119** results in a marked decrease in the association constant between the modified S-Peptide or S-Protein and its native partner. In either case addition of the unmodified component results in rapid and complete displacement of the damaged member and restoration of activity. Thus both of these histidyl residues are intimately involved with the structural integrity of the functioning enzyme.

Both iodination and ring destruction of histidine-**12** lower the association constant by a factor of the order of 1000. Thus histidine-**12** as well as methionine-**13** (25) is intimately involved in the association process.

This conclusion on the structural role of histidine-**12** has been drawn previously by Yang and Hummel (27) in their study of the denaturation of alkylated derivatives of the native enzyme. The actual changes in atomic coordinates required to explain a change in binding constant of a factor of 1000 cannot be stated with any certainty, but changes of a few tenths of an angstrom unit might easily be expected. While there are residues which can be shown to have solely a structural role (*i.e.* methionine-**13**), it appears likely that all residues directly involved in catalysis will also contribute importantly to structure and stability. For any enzyme there is assumed to be an accurately maintained geometry at the active site. Specific residues held in the proper orientation by the rest of the protein structure must, of course, themselves contribute to the integrity of that structure.

The data in Fig. 1 clearly imply protection of histidine-**12** in RNase-S. This residue may be partly buried as is the case with some members of the same class of residues in myoglobin (28). In any event, its environment must be different from residue **119**. In this sense the relative behavior is the same as in the reaction with iodoacetate ion where again **119** is more reactive than **12** (29). However, in the latter reaction histidine-**119** has an unusually high reactivity which is intimately connected with the enzymically active three-dimensional structure (30). Residue **12** is also more active than normal although less reactive than **119**. In the photooxidation reaction **119** and **105** appear to react at comparable rates, while **12** has a rate much lower than "normal." The data of Heinrikson *et al.* (6) show an inversion of relative reactivity between **12** and **119** for certain alo acids. In view of these latter observations, any definite conclusions as to the degree of "exposure" of **12** and **119** on the surface of the enzyme will require some other type of experimental approach.

An additional difference between the photooxidation and alkylation reactions is revealed by the substrate effect. The rate of inactivation of the enzyme by iodoacetate ion is decreased in the presence of substrate, whereas no such effect was observed in the photooxidation reaction. The substrate was tested at a concentration about equal to the Michaelis constant, therefore half of the enzyme should have been in the free form. Reduction of the inactivation rate by as much as a factor of 2 thus might have been expected. However, there was no detectable change. Since the same residues are affected in the two different reactions, the use of a substrate protection effect to localize a residue at the active site should be approached with caution. For the existence of this effect it is necessary, although not sufficient, that the rate-limiting process be the bimolecular step between reagent and reactive residue. The mechanism of the sensitized photooxidation process has many steps (31) and it is quite possible that the particular bimolecular step involving the histidine residue is not, in fact, rate-limiting. The difference in size between the iodoacetate ion and the singlet oxygen molecule may also, of course, bear on the difference in the substrate effect for the two reactions.

The 3-carboxymethylhistidine-**12** derivatives of RNase-A appears to have little or no enzymic activity towards cytidine cyclic phosphate (29). The 2(4)-iodohistidine-**12**-S-Peptide derivative described in this paper shows no activity when mixed with S-Protein. The binding of this last derivative is markedly reduced compared to S-Peptide but is quite sufficient to easily demonstrate complex formation under assay conditions. The photooxidized peptide is also inactive, as discussed above. The only discrepancy at the moment is the report by Vithayathil and Richards (25) that 3-carboxymethylhistidine-**12** $MetO_2$-S-Peptide has the same potential activity as $MetO_2$-S-Peptide itself. These results were obtained on a rather impure mixture of peptides which was not fully characterized, and RNA was used in the assay as a substrate rather than the cyclic phosphate. It is clear that better preparations and comparable assays are required.

The photooxidation reaction in solution and in the crystals appears to take place in a similar fashion. The same residues are affected in each case and the protection of histidine-**12** is also apparent in both cases. The only marked difference is the time required for the reaction to occur, the crystals being about 50 times slower than the solution. The ionic strength is, of course, markedly different, but all other solvent conditions are very similar. There may be a direct ionic strength effect on the reaction. In addition, the very high salt concentration will lower the solubility of oxygen. It seems likely that some general effect of this latter type is responsible for the rate difference. A change in conformation of the protein between crystal and solution would hardly be expected to have the same relative effect on 3 different histidine residues. Thus the conclusion of Doscher and Richards (11), on the basis of activity measurements, that the solution and crystal structures of RNase-S must be very similar is apparently supported by this quite different type of reaction.

The conclusion just given is contradicted, or at least rendered uncertain, by the recent very interesting report by Winstead and Wold (32) on the effect of high salt concentration on the kinetic properties of RNase. Marked changes in absolute and relative activity toward RNA and cyclic cytidylate were observed in the

range of salt concentration of 0 to 4 M. Various salts and RNase derivatives were studied. The authors conclude that there is a difference between the structures of the enzyme in dilute salt solution and in concentrated ammonium sulfate. The implication is clear that even if no change in protein structure occurred on crystallization from strong salt solutions, there would still be a difference between the crystal structure and that in dilute salt solution. The nature and extent of the change cannot be specified at this time.

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