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Dye-sensitized Photooxidation As a Tool for Determining the Degree of Exposure of Amino Acid Residues in Proteins

THE METHIONYL RESIDUES IN RIBONUCLEASE A*

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SUMMARY

Optical rotation measurements and absorption difference spectroscopy of ribonuclease A in different water-acetic acid mixtures showed that at least three conformational situations exist for the protein molecule at different levels of acetic acid concentration. Hematoporphyrin-sensitized photooxidation of the native as well as of the denatured proteins allowed us to detect three discrete states of reactivity for the 4 methionyl residues of ribonuclease A. On the basis of the photooxidation kinetics and of the conformational analyses, we concluded that methionine-29 is partly exposed in the native protein and methionine-13 is partially buried, whereas methionine-30 and methionine-79 are deeply buried. The importance of the single methionyl residues for the catalytic activity of the enzyme has been also resolved. The described procedure appears to be a reliable tool for examining the state of the amino acid residues in proteins.

The dye-sensitized photochemical modification of proteins is a very active area of research (1, 2). Usually, the aim of such studies is to perform the photooxidation of specific amino acid residues and to determine the resultant changes in the biological, physical, and chemical properties of the protein. In the early 1960's, new prospects were introduced into this field by Ray and Koshland (3). These authors demonstrated that the drop in the sensitivity to the photodynamic treatment displayed by some amino acids in proteins, relative to that in small peptides, is due to the restrictions imposed by the protein conformation to the contact between the side chains and the photoexcited sensitizer. This resulted in the subdivision of the photooxidizable amino acid residues into normal (fully exposed) and abnormal (buried), and opened the way to the probing of the three-dimensional structure of proteins by means of dye-sensitized photooxidation.

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However, it has already become clear that such a sharp classification into completely accessible and completely inaccessible residues is an oversimplification, since both spectroscopic (4) and chemical modification (5) studies pointed out that many amino acid side chains belong to intermediate classes, neither fully buried nor fully exposed. Therefore, in order to enlarge the scope of the aforesaid technique, it would be desirable to develop more refined procedures, allowing one to detect the occurrence of any intermediate degree of exposure.

In this paper, we propose a method which should, at least in part, circumvent some of the drawbacks inherent to the procedure of Ray and Koshland. It consists of inducing a progressive, multistage unfolding of a protein molecule by the stepwise addition of a suitable denaturing agent. Then the protein molecules corresponding to the different denaturation states are subjected to dye-sensitized photooxidation. In this way, a gradually increasing number of amino acid residues should become susceptible to photooxidation: first the fully exposed residues, then—in subsequent steps—the partially buried residues, and finally the deeply buried residues.

The validity of this hypothesis is clearly shown by our findings about the degree of burial of the methionyl residues in RNase A.[1] This protein has been chosen as a model for a first study since its three-dimensional structure in the crystal state, as well as that of its strictly related derivative RNase S, has been already resolved to an atomic detail by x-ray crystallography (6, 7); consequently, suitable data for comparison with our results were available. The procedure adopted was the hematoporphyrin-sensitized photooxidation, since this dye has been recently shown (8) to act selectively on the methionyl residues under a large spectrum of experimental conditions.

EXPERIMENTAL PROCEDURE

Materials—RNase A (Grade V, Seravac Laboratories) was purified by column chromatography (9) on Amberlite CG-50 (British Drug Houses, London). The concentration of the

[1] The abbreviations used are: RNase A, the principal chromatographic component of bovine pancreatic ribonuclease; RNase S, subtilisin-modified RNase A, from which S-protein and S-peptide (residues 1–20) are obtained; Z-Met-Asp, *N*-benzyloxycarbonyl-L-methionyl-L-aspartic acid; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP-, 2,4-dinitrophenyl-.

protein solutions was estimated spectrophotometrically on the basis of the extinction value $E^{1\%}_{1\,cm} = 6.95$ at 280 mμ (10). L-Methionine was obtained from Fluka AG (Basel, Switzerland). The peptide *N*-benzyloxycarbonyl-L-methionyl-L-aspartic acid was synthetized in this Institute. Hematoporphyrin dihydrochloride was a product of Fluka and appeared to be homogeneous by thin layer chromatography in different solvent systems. 1-Fluoro-2,4-dinitrobenzene, purchased from E. Merck (Darmstadt, Germany), was distilled under reduced pressure prior to use. Cyanogen bromide (Schuchardt) and 95% acetic acid (E. Merck) were used as received. Sephadex G-25, medium grain size, was supplied by Pharmacia (Uppsala, Sweden). Trypsin, twice crystallized, was obtained from Worthington and was used without further purification. Commercial yeast RNA (sodium salt) was purchased from Schwarz Laboratories and purified by exhaustive dialysis against 0.1 M sodium chloride and then against cold distilled water. All other chemicals were purchased commercially and were reagent grade products, unless otherwise stated.

Procedure of Photooxidation—In a typical experiment, 4 ml of a 1 mM protein solution, added in the dark with an equimolar amount of hematoporphyrin, were exposed to the light of four 300-watt tungsten bulbs placed on either side of a water bath with Plexiglas walls. The irradiation apparatus and the experimental procedure were detailed elsewhere (8, 11). The temperature was maintained at 25° ± 0.1°. During irradiation, a stream of purified oxygen was slowly flushed through the solutions. Controls without oxygen or light were also run. At the end, the photooxidized samples of RNase A were freed of the sensitizer by gel filtration through a column (0.9 × 48 cm) of Sephadex G-25, with 0.2 M acetic acid as the eluent. The protein was recovered by lyophilization. The photooxidation of methionine and of Z-Met-Asp was performed under conditions identical with those described above.

Amino Acid Analyses—The amino acid content of the unirradiated and of the photooxidized samples of RNase A was evaluated by subjecting the protein to hydrolysis by 6 N HCl within evacuated sealed vials at 110° for 22 hours. The dried hydrolysate was chromatographed on a Carlo Erba 3A27 automatic analyzer according to the procedure of Spackman, Stein, and Moore (12). The results obtained were standardized against valine, leucine, and phenylalanine since these amino acids are known to be unaffected by photooxidation (1) and are also stable to acid hydrolysis. The content of methionine and and of methionine sulfoxide was determined quantitatively after alkaline hydrolysis in 3.75 N NaOH (13).

The kinetics of methionine and of Z-Met-Asp photooxidation was followed according to the procedure previously outlined (8). In the case of RNase A, the time course of methionine photooxidation was determined by monitoring on the amino acid analyzer the amount of methionine remaining in samples of protein irradiated for different periods of time.

Reaction with Cyanogen Bromide and Amino End Group Analysis—The reaction of the photooxidized samples of RNase A with cyanogen bromide was carried out by essentially the procedure of Gross and Witkop (14), with the exception that 70% formic acid was used as the solvent in the place of 0.1 N HCl.

The amino end group analyses of the CNBr-treated products were brought about by reaction with FDNB according to the method of Sanger (15); the DNP-amino acids were identified by paper chromatography on Whatman No. 1, with the solvent systems described by Levy (16). Known amounts of reference solutions of DNP-amino acids were chromatographed simultaneously with the analyzed samples. For the quantitative determinations, the spots were cut out of the paper in strips of identical size and eluted with 1% $NaHCO_3$; the optical density of the resulting solution was read at 350 mμ. Identical areas of unstained sheet were subjected to the same elution procedure and were used as a blank for the spectral readings.

Chromatographic Analyses—The samples of native and photooxidized RNase A were chromatographically analyzed on a column (0.9 × 53 cm) of Amberlite CG-50. In all cases, the column was loaded with about 5 mg of protein and was operated at pH 6.45 under the conditions described by Hirs, Moore, and Stein (9). Aliquots from each column fraction were used to measure the optical density at 277 mμ and to develop ninhydrin color after adjustment of the pH to 5.5 ± 0.2 with 4 M sodium acetate buffer.

Tryptic Hydrolyses—The tryptic hydrolyses of native and photooxidized RNase A were performed at 25° with a trypsin to protein ratio of 1:10 by weight and an RNase A concentration of 0.5%. The pH of the solution was maintained at 7.8 ± 0.1 by the addition of 0.01 N NaOH. The alkali consumption was followed with an autotitrator (Radiometer, Copenhagen, type TTT1c) equipped with a combined calomel electrode.

Spectral Measurements—Absorption values at single wave lengths were obtained with an Hitachi-Perkin Elmer model 139 spectrophotometer. Continuous spectra were recorded by means of an Optica CF 4 DR spectrophotometer. Difference spectra were recorded in the Optica instrument with two matched 1-cm quartz cuvettes containing protein solutions of equal concentration. The previously described precautions were observed in order to minimize any artifact due to stray radiation or to fluorescence of protein solutions (17, 18).

The measurements of optical rotation were carried out at 25° and at 365 mμ by means of a Perkin Elmer model 141 polarimeter with a mercury arc as the light source. In all cases, 0.2% solutions of protein were introduced into a 1-cm jacketed quartz cell. The temperature dependence of the rotatory power was studied by the procedure previously described (17).

Circular dichroism measurements were performed with a Roussel-Jouan CD 185 dichrograph with nitrogen flushing below 200 mμ. The protein samples were examined at concentrations from 0.5 to 1.8 mg per ml. Cylindrical Opticell quartz cells were used with 1-, 0.1-, 0.05-, and 0.01-cm optical path. All measurements were made at room temperature. The absence of optical artifacts was determined by recording the spectrum of the same sample in cells of different optical path. The data are expressed in terms of $[\theta]_\lambda$, the mean residue molecular ellipticity, defined as $[\theta]_\lambda = 3300\ (\epsilon_l - \epsilon_r)$ deg cm² dmole^{-1}, where $(\epsilon_l - \epsilon_r)$ is the difference between the molar (on a mean residue basis) extinction coefficients for left and right circularly polarized light.

Enzymic Activity—The enzymic activities of photooxidized RNase A were determined by the spectrophotometric procedure of Kunitz (19), with RNA as a substrate. A solution of native RNase A at the same protein concentration was always assayed along with the photooxidized samples.

RESULTS

Conformational Studies on Native RNase A—At first, we examined the stability of native RNase A to the action of low pH values and of acetic acid. These denaturing agents were

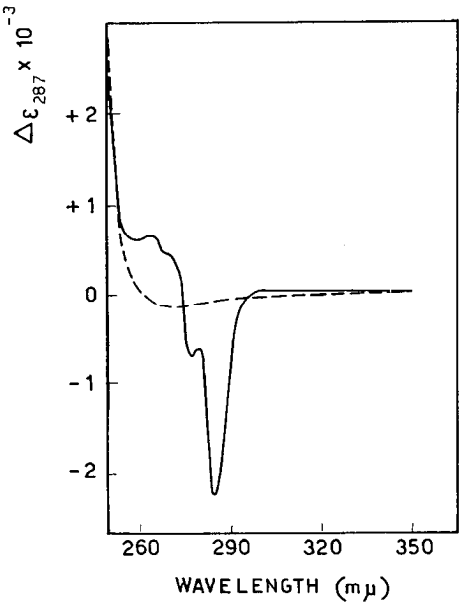

FIG. 1. Difference absorption spectrum obtained by reading a 1.8×10^{-4} M solution of RNase A in 80% acetic acid against an equimolar solution of the same enzyme in distilled water, pH 5.95. The *dotted line* represents the spectrum obtained by reading 80% acetic acid against water.

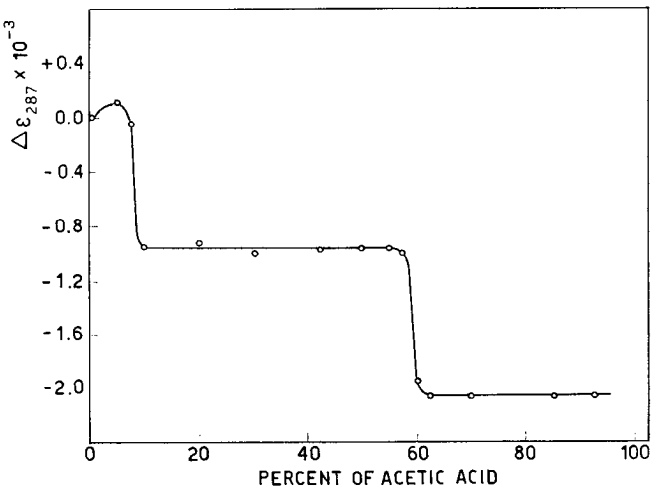

FIG. 2. The effect of the acetic acid concentration on the molar difference extinction coefficient of RNase A at 287 mμ. The *single points* were determined under the conditions described in the legend to Fig. 1.

selected since they can be varied by microamounts over a sufficiently wide range: in this way, the occurrence of any intermediate stage of unfolding might be detected. Moreover, acetic acid has been shown to be a very good solvent for the dye-sensitized photooxidation of amino acids and proteins (8, 13, 20). On the contrary, other widely used denaturants, such as urea or guanidine hydrochloride, may unfavorably interfere with the photochemical process, either by being competitively photooxidized or by quenching the excited states of the sensitizer (1, 2). As a probe for the conformation of both the peptide backbone and the amino acid side chains, we used polarimetry and difference absorption spectroscopy since these techniques are known to be very sensitive to even small conformational changes of protein molecules (21, 22).

Actually, the optical parameters of RNase A displayed only minor variations when the pH of the solution was lowered to pH 2 by the stepwise addition of HCl. This is in agreement with the findings of previous investigators (23, 24), who established that the molecule of RNase A is well resistant to moderately acidic aqueous media.

On the contrary, dramatic effects appeared to occur as soon as the enzyme was exposed to concentrated acetic acid. In Fig. 1, we report the difference spectrum obtained by reading a 0.18 mM solution of RNase A in 80% acetic acid against an equimolar solution of the protein in distilled water, pH 5.95. The most interesting feature of the spectrum is the negative absorption maximum centered at 287 mμ; this clearly reflects a blue shift in the absorption of the tyrosyl chromophores and may be interpreted as due to the disruption of the secondary and tertiary structure of the protein molecule and to the consequent exposure of the formerly buried tyrosyl side chains to the water-acetic acid environment (25). The observed drop in the molar extinction is both qualitatively and quantitatively analogous to that found by Bigelow and Geschwind (26) when RNase A was incubated for several minutes in the presence of high concentrations of urea, that is, in a very effective denaturing medium. Therefore, it appears reasonable to assume that the molecule of RNase A is extensively disorganized by 80% acetic acid.

The depression in the absorption parameters of the enzyme caused by acetic acid takes place in two distinct steps. Actually, if one plots the $\Delta\epsilon_{287}$ against the concentration of acetic acid, three plateaus can be distinguished between 0 and 5%, 10 and 55%, and over 62% acetic acid (see Fig. 2). This would suggest that at least three different conformational situations of RNase A can exist at different levels of acetic acid concentration.

Further support to this conclusion was lent by optical rotation measurements. As shown in Fig. 3, increasing the percentage of acetic acid resulted in a progressive increase in the levorotation. With subsequent heating, the thermally induced transition occurred at progressively lower temperatures, and there was a reduction in the amount of heat-induced rotatory change observed.[2] Once again, the decrease in the midpoint of the thermal transition took place within critical ranges of denaturant concentration, which closely corresponded to those previously observed for the decrease in the extinction at 287 mμ. In particular, in 5% acetic acid solution, the transition temperature of RNase A was 64°, almost identical with that determined by Sherwood and Potts (10) for the enzyme in phosphate buffer, pH 6.8; this shows that, up to this concentration of acetic acid, there is no significant perturbation of the native structure of the protein. Between 10 and 55% acetic acid, the levorotation slightly increased, but the midpoint of the thermal transition was constantly found at 53° ± 2°, indicating that these amounts of acetic acid induce about the same degree of loosening of the molecule of RNase A. Finally, above 62% acetic acid, only a barely detectable thermal transition was present, the transition temperature being located at approximately 40°. Therefore, in this medium, RNase A must retain very little ordered structure.

Photooxidation Studies—In the light of the previous con-

[2] No time-dependent effects were observed once the solutions had reached thermal equilibrium, and heating the solvent alone produced no appreciable changes in rotation.

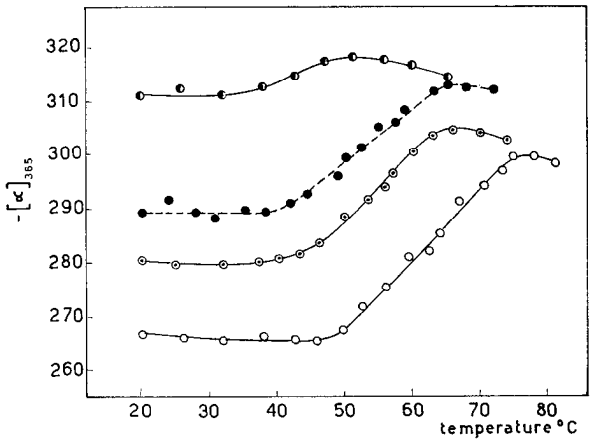

FIG. 3. The effect of temperature on the specific rotation at 365 mμ of RNase A in different water-acetic acid mixtures: O——O, 5% acetic acid; ⊙——⊙, 10% acetic acid; ●- - -●, 55% acetic acid; ◐——◐, 64% acetic acid. In all cases, 0.2% solutions of protein were used.

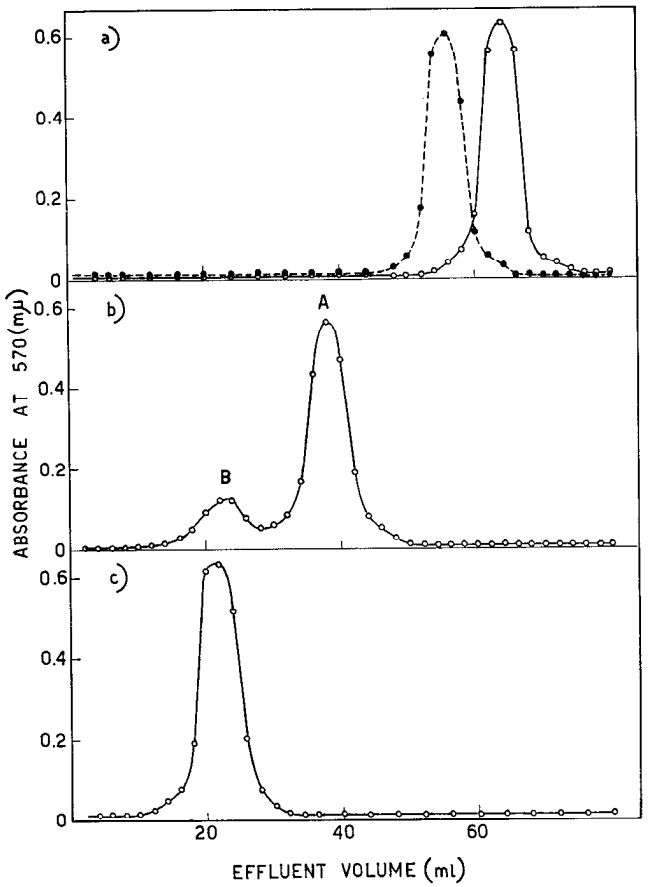

FIG. 4. Chromatograms of native and photooxidized samples of RNase A on an Amberlite CG-50 column (0.9 × 53 cm). The eluting buffer was 0.2 M sodium phosphate, pH 6.45. *a*, native RNase A (O——O) and RNase A irradiated for 10 min in 5% acetic acid (●- - -●); *b*, RNase A irradiated for 10 min in 10% or in 50% acetic acid; *c*, RNase A irradiated for 10 min in 67% acetic acid.

formational investigations, distilled water (pH 5.9) and 5, 10, 50, and 67% acetic acid were selected as solvents for running the photooxidation of RNase A sensitized by hematoporphyrin. In the experiments in water, there was no detectable change

TABLE I

Amino acid analyses of unirradiated and of photooxidized[a] RNase A

The amino acids were determined chromatographically on a Carlo Erba 2A27 amino acid analyzer after 22-hour hydrolysis in 6 N HCl at 110°. The table includes only those amino acids which are known to be affected by photooxidation (1). No appreciable change was found in the other amino acids analyzed.

Photooxidation medium	Amino acid content			
	Histidine	Tyrosine	Methionine[b]	Methionine sulfoxide[b]
	residues/molecule			
Distilled water (pH 5.9)[c]	3.8	5.9	2.9	1.0
5% acetic acid	4.0	6.2	3.1	1.0
10% acetic acid	3.8	5.7	1.7	2.2
50% acetic acid	3.8	5.7	1.9	2.1
67% acetic acid	3.9	5.8	0.0	3.8
Unirradiated protein	3.8	5.8	4.1	0.0

[a] All irradiations were carried out for 10 min at 25° ± 0.1°.

[b] Evaluated by automatic chromatographic analysis after 14-hour hydrolysis at 100° in 3.75 N NaOH (13).

[c] The pH of the solution did not change during irradiation.

of the pH during irradiation. The progress of the reaction was followed by column chromatography on the carboxylic acid resin Amberlite CG-50. The elution patterns obtained after 10 min of irradiation are reported in Fig. 4.

Up to 5% acetic acid, one peak was observed (Fig. 4*a*), which partially overlapped the peak of native RNase A. However, the presence of any unreacted enzyme was ruled out since (*a*) both the retention volume and the shape of the peak did not change if the illumination was prolonged for 1 hour and (*b*) the enzymic activity, ultraviolet absorption spectrum, and rotatory power of the material isolated from the fractions in the leading slope of the peak were coincident with those of the product constituting the whole peak.

A single peak was also observed after chromatography of solutions irradiated in 67% acetic acid (Fig. 4*c*). This sample moved considerably ahead of the native protein, indicating that, after photooxidation in this milieu, the basic net surface charge of RNase A was appreciably lowered. The R_F of the peak was unaffected by further exposure to light.

When the irradiations were carried out in 10% or in 50% acetic acid, *i.e.* in correspondence of the intermediate plateau, a second minor peak (Peak *B*) was invariably present in addition to the main Peak *A* (Fig. 4*b*): its retention volume was identical with that of the peak observed after photooxidation of RNase A in 67% acetic acid solution. Increasing the irradiation time caused a progressive slow conversion of Peak *A* to Peak *B*. It thus appears that the product corresponding to Peak *A* is susceptible to further photooxidation under these conditions of irradiation. However, the low rate of its photodestruction allowed us to recover amounts of this sample sufficient for further characterization.

The amino acid content of the products obtained in the single photooxidation experiments is shown in Table I. As expected (8), no amino acid residue other than methionyl was modified under these conditions. Moreover, a close correspondence appeared to exist between the acetic acid-induced conformational transitions of RNase A and the susceptibility of the methionyl residues to the photodynamic treatment. Actually 1 methionyl

TABLE II

Identification of methionyl residues photooxidized in different reaction media

The identification was based on the nature and on the amount of NH_2-terminal amino acids which were present after reaction of the photooxidized protein with CNBr (see text).

Irradiation medium	Methionyl residues modified per molecule of protein[a]	DNP-amino acids recovered[b]	Identity of the photooxidized methionyl residues[c]
		residues/molecule	
0 or 5% acetic acid	1	Di-DNP-Lys (1.76) DNP-Asp (0.73) DNP-Ser (0.84)	Met-29
10 or 50% acetic acid	2	Di-DNP-Lys (1.80) DNP-Ser (0.79)	Met-29, Met-13
67% acetic acid	4	Di-DNP-Lys (0.84)	Met-29, Met-13 Met-30, Met-79

[a] Evaluated by amino acid analysis (see Table I).

[b] Evaluated by optical density reading at 350 mμ after chromatographic separation on paper of the DNP-amino acids and subsequent elution of the yellow spots from the paper with 1% $NaHCO_3$.

[c] Partial amino acid sequences around the methionyl residues: His_{12}–Met_{13}–Asp_{14}; Gln_{28}–Met_{29}–Met_{30}–Lys_{31}; Thr_{78}–Met_{79}–Ser_{80}.

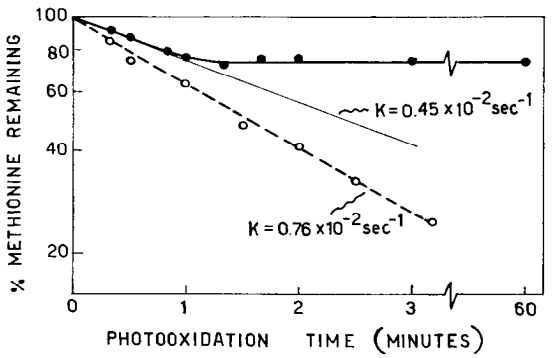

FIG. 5. Time course of the hematoporphyrin-sensitized photooxidation of L-methionine (O- - -O) and of the methionyl residues in RNase A (●——●) in 5% acetic acid solution at 25°. The reaction mixture was 1 mM in both the substrate and the sensitizer.

residue per protein molecule was converted to the sulfoxide by photooxidation in up to 5% acetic acid, 2 methionyl residues were photooxidized between 10% and 50% acetic acid, and all 4 methionyl residues which are present in RNase A became susceptible in 67% acetic acid. Both the mono- and the tetrasulfoxide derivatives of RNase A were resistant to prolonged irradiation; on the contrary, the disulfoxide RNase A, if further subjected to hematoporphyrin-sensitized photooxidation in either 10% or 50% acetic acid, underwent a gradual conversion to the tetrasulfoxide derivative. This result is in agreement with the chromatographic data.

The location of the photooxidized methionyl residues in the amino acid sequence of RNase A was identified by allowing the irradiated samples to react with cyanogen bromide. This reagent selectively cleaves a polypeptide chain at the carboxyl end of the methionyl residues, but not of the methionine sulfoxide residues (14). Consequently, new NH_2-terminal amino acids should be formed only in correspondence with the unmodified

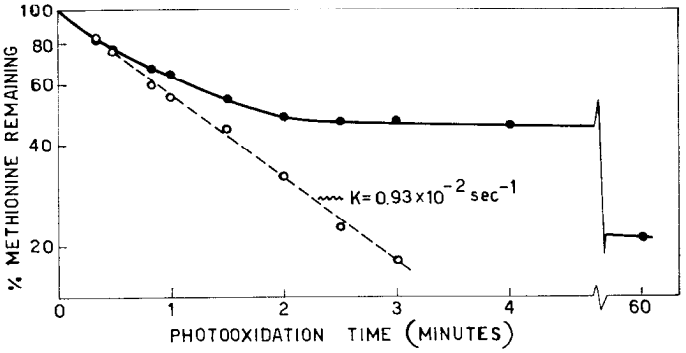

FIG. 6. Time course of the hematoporphyrin-sensitized photooxidation of L-methionine (O- - -O) and of the methionyl residues in RNase A (●——●) in 50% acetic acid solution. All other conditions were the same as in Fig. 5.

methionyl residues. A comparison of the amino acid sequences with the data on the recovery of the DNP-amino acids from the CNBr-treated products, reported in Table II, allows one to identify the photooxidized residues in an unequivocal fashion.

Clearly, methionine-29 is the residue which is specifically modified when RNase A retains its native conformation. This conclusion was further supported by gel filtration of the CNBr-treated monosulfoxide derivative on Sephadex G-25: no trace could be detected of homoserine lactone or of homoserine which should be released by cleavage at both sides of methionine-30 (14). Methionine-13, on the other hand, becomes available for photooxidation upon partial unfolding of the protein molecule; finally, methionine-30 and methionine-79 remain inaccessible to the photooxidizing agent until the tertiary structure has been extensively disorganized. It may be noted that the recoveries of the DNP-amino acids were somewhat lower than the theoretical ones; this was probably due to incomplete cleavage of the polypeptide chain of RNase A by cyanogen bromide, as found also by other authors (27, 28).

Kinetic Studies—The time course of the hematoporphyrin-sensitized photooxidation of methionine is first order with respect to the amino acid both in aqueous and in acetic acid solution (8).

Now, in all of the reaction media studied by us, the photooxidation kinetics of free methionine was coincident, within experimental error, with that of the methionyl residue in Z-Met-Asp: this shows that masking the α-amino group and α-carboxyl group has no effect on the photoreactivity of this amino acid.

In Fig. 5, the kinetics of photooxidation of the methionyl residues in RNase A in 5% acetic acid solution is compared with that determined for the free methionine under the same conditions. The decrease in the methionine content of the protein follows a first order kinetics only for about 1 min and then levels off at about 75% of remaining methionine. This was to be expected since amino acid analysis pointed out that only 1 out of the 4 methionyl residues was converted to methionine sulfoxide in this milieu. However, the rate constant, as calculated from the slope of the linear portion of the curve, *i.e.* 0.45×10^{-2} sec^{-1}, was appreciably lower than that found for free methionine, *i.e.* 0.76×10^{-2} sec^{-1}.

A more complex plot was obtained when the time course of the photooxidation of the methionyl residues in RNase A was determined in 50% acetic acid solution (see Fig. 6).

Similar plots were obtained in 10% acetic acid. Apparently, the curve can be divided into two portions, reflecting the oxida-

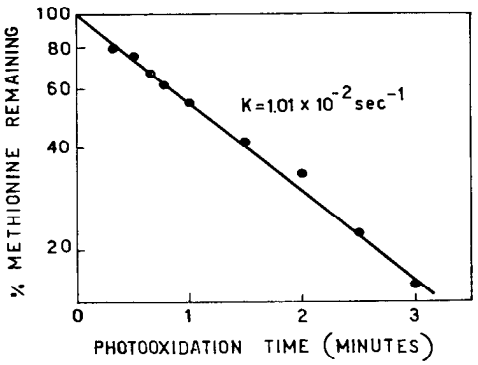

FIG. 7. Time course of the hematoporphyrin-sensitized photooxidation of the methionyl residues in RNase A in 67% acetic acid solution. The conditions of irradiation were the same as in Fig. 5. The kinetic plot for free methionine was coincident with that reported in the figure, within experimental error.

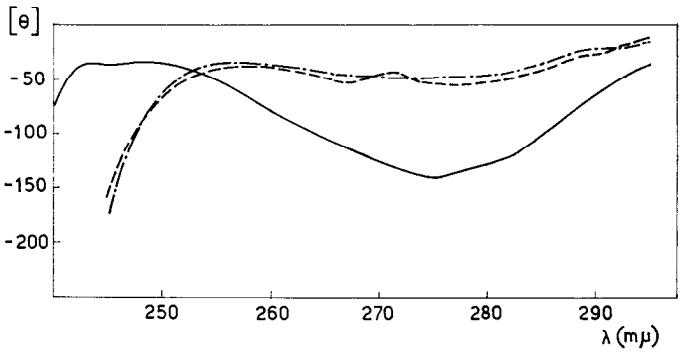

FIG. 8. Near ultraviolet circular dichroism spectra of methionine$_{29}$ sulfoxide-RNase A (——), methionine$_{29}$ sulfoxide-methionine$_{13}$ sulfoxide-RNase A (– – –), tetrasulfoxide RNase A (–·–·–). All solutions were made in water at protein concentrations of 0.1 to 0.15%.

TABLE III

Some properties of native RNase A and of its mono- and polysulfoxide derivatives

Sample	Peptide bonds cleaved by tryptic digestion	$-[\alpha]^{25}_{365}$ (c = 0.2% in water)	Absorption maximum	Enzymic activity
			mμ	%
Unirradiated	1.5	266	277.5	100
Methionine$_{29}$ sulfoxide-RNase A	2.2	273	277.5	65
Methionine$_{29}$ sulfoxide-methionine$_{13}$ sulfoxide-RNase A	9.7	299	276.0	17
Tetrasulfoxide RNase A	10.9	312	276.0	13

tion of a fast reacting group of methionyl residues (from 0 to about 2 min) and of a slow reacting group (above 2 min).

Graphic analysis of this curve according to the procedure of Ray and Koshland (3) suggests that about 50% of the methionyl residues, *i.e.* 2 out of 4, are involved in the slow reacting group. Again, this is consistent with the data from amino acid and chromatographic analysis, showing that the originally formed disulfoxide RNase A is slowly converted to the tetrasulfoxide derivative. On the other hand, the first portion of the curve displays an exponential shape except during the initial 30 sec, during which the semilogarithmic plot is linear and coincident with the experimentally determined plot for free methionine.

When RNase A was photooxidized in 67% acetic acid, the decrease in the methionine content followed a first order kinetics at least down to 90% of conversion (Fig. 7), indicating that all 4 methionyl residues were oxidized at the same rate. The monomolecular rate constant was 1.01×10^{-2} sec^{-1}, and was practically coincident with that found for the photooxidation of free methionine under the same conditions.

Properties of Products of RNase A Photooxidation—In Table III we summarize some properties of native RNase A as well as of its mono- and polysulfoxide derivatives.

As expected, native RNase A was very resistant to proteolysis by trypsin (29), only about 1.5 out of the possible 15 bonds being split. A rather similar behavior was displayed by methionine$_{29}$ sulfoxide-RNase A. On the contrary, both the di- and the tetrasulfoxide proteins were highly susceptible. Significantly, the percentage of peptide bonds hydrolyzed in tetrasulfoxide RNase A was almost identical with that found for RNase A in which the disulfide bridges had been reduced and carboxymethylated (30). It appears that a collapse of the native structure of the enzyme must have occurred after photooxidation of methionine-13.

These findings were paralleled by the measurements of optical rotation at 365 mμ. Whereas methionine$_{29}$ sulfoxide-RNase A showed only a slight increase in levorotation with respect to the unirradiated protein, both the di- and the tetrasulfoxide derivatives possessed a much higher specific rotation. A similar increase in this parameter was observed by Sherwood and Potts (10), after exposure of RNase A to high concentrations of urea. Furthermore, the ultraviolet absorption spectrum of methionine$_{29}$ sulfoxide-RNase A was superimposable on that of the native enzyme; on the contrary, both the di- and tetrasulfoxide derivatives were characterized by a small but distinct shift in the absorption maximum from 277.5 mμ to 276 mμ: this corresponds to the well known "denaturation blue shift" which is typical of unfolded RNase A (26, 31), and results from alterations in the environment of the tyrosyl residues.

More definite conclusions could be drawn by monitoring the circular dichroism spectra of the single photooxidation products.

As one can see from the aromatic region of the dichroic curves (Fig. 8), more and more marked changes in the environment of the tyrosyl residues were introduced as the number of the photooxidized methionyl residues was increased. Actually, the loss of the small positive band around 240 mμ, which is characteristic of native RNase A (32), and the reduction in the intensity of the negative band are typical of denatured RNase A (32–34).

The far ultraviolet dichroic spectra (Fig. 9), which mainly reflect the situation of the peptide backbone, give the same kind of evidence. The curve of methionine$_{29}$ sulfoxide-RNase A retains the main features typical of the spectrum of the native protein (35). On the contrary, the curves of the di- and tetrasulfoxide RNase A are typical of largely disordered proteins. This is especially evident owing to the presence of an intense extremum around 202 mμ, which is predominantly originated by the amide $\pi \rightarrow \pi^*$ transition associated with the disordered form of proteins (36).

Finally, the photooxidized samples were tested for their enzymic activity toward RNA. As shown in Table III, Column 4, the monosulfoxide derivative maintained a high catalytic

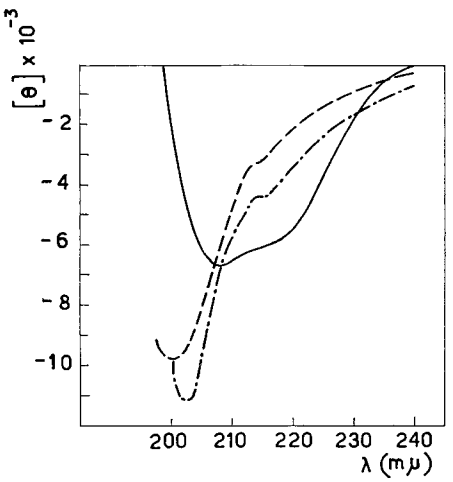

FIG. 9. Far ultraviolet circular dichroism spectra of methionine$_{29}$ sulfoxide-RNase A (——), methionine$_{29}$ sulfoxide-methionine$_{13}$ sulfoxide-RNase A (– – –), tetrasulfoxide RNase A (–·–·–). All solutions were made in water at protein concentrations of 0.1 to 0.15%.

efficiency; on the contrary, after photooxidation of methionine-13, there was a dramatic drop in the enzymic activity to 17% with respect to the native protein. Oxidation of methionine-30 and methionine-79 caused a slight further decrease of the enzymic activity to 13%. This value is coincident with that previously found for the tetrasulfoxide RNase A, prepared by methylene blue-sensitized photooxidation in 84% acetic acid solution (13).

DISCUSSION

Our results show that there are three discrete states of photoreactivity for the methionyl residues in RNase A.

Methionine-29 is sensitive to the photodynamic action of hematoporphyrin even when the protein retains its native conformation. Therefore, this residue appears to be exposed to the solvent in the RNase A molecule. However, the analysis of the photooxidation kinetics suggests that the exposure of this residue is not complete. Actually, the rate constant for the photooxidation of methionine-29 in 5% acetic acid (0.45×10^{-2} sec^{-1}) is appreciably lower than that found for free methionine or for Z-Met-Asp (0.76×10^{-2} sec^{-1}) under the same conditions. In 67% acetic acid solution, *i.e.* when RNase A is extensively unfolded, there is no difference between the photooxidation rate of the methionyl residues in the protein and that of the free amino acid. This means that the drop in photoreactivity displayed by methionine-29 in 5% acetic acid is not due to the incorporation into the polypeptide chain by itself, but to restrictions imposed by the three-dimensional conformation of the protein molecule to the interaction between methionine-29 and the reactive photooxidizing agent.

In any case, the conclusion that methionine-29 is located near the surface of the molecule is in agreement with the fact that only limited alterations in the over-all spatial geometry of RNase A take place when methionine-29 is converted to the sulfoxide, as shown by the physicochemical and enzymic properties of methionine$_{29}$ sulfoxide-RNase A. Therefore, the side chain of methionine-29 must be only little involved in the intramolecular forces which stabilize the three-dimensional structure of RNase A. Of course, the introduction of a hydrophilic center (the S=O group) into the hydrophobic side chain of methionine-29 may be expected to bring about some local perturbations in the microenvironment of this residue. Actually, in the circular dichroism spectrum of methionine$_{29}$ sulfoxide-RNase A, the 240 mμ band, which is highly sensitive to conformation (32, 34, 37), is missing; moreover, the rotational strength of the longer wave length dichroic band is slightly depressed.

Our findings receive some support from the literature. For example, Link and Stark (28) succeeded in selectively alkylating methionine-29 by reaction of RNase A with methyl iodide at pH 2.5 to 5.0; in this case too, the rate of methylation of this residue was about 45% of that of *N*-acetylmethionine. Moreover, Neumann, Moore, and Stein (38) treated RNase A with hydrogen peroxide in slightly acidic solutions and isolated an enzymically active monosulfoxide derivative. In the light of the present results, it appears reasonable to identify this product as methionine$_{29}$ sulfoxide-RNase A. Finally, the x-ray structure of RNase S at 2 A resolution (7) shows that methionine-29 is at least partially exposed.

A partial loosening of the native molecule of RNase A is necessary in order that methionine-13 becomes susceptible to photooxidation. However, the initial portion of the plot for the decrease in the methionine content of RNase A as a function of the irradiation time deviates from linearity, suggesting that 1 or both of the photooxidizable methionyl residues are not fully "free." Since methionine-29 is already exposed in the native protein, the nonlinearity of the kinetic plot can be conceivably ascribed to the fact that the opening of the RNase A molecule is not large enough to expose the side chain of methionine-13 in a complete fashion.

Previous investigations (39–41) emphasized that methionine-13 plays an important structural role in RNase S, since any introduction of a polar center in the place of the apolar thioether function of this residue drastically reduces the binding affinity of the S-peptide for the S-protein. However, this residue is not directly involved in the catalytic action of the enzyme (40). Consequently, it is quite likely that the abrupt fall in the catalytic efficiency of RNase A, which is observed after photooxidation of methionine-13, is due to dramatic distortions of the conformation of the molecule.

Actually, all of the conformational parameters determined, and in particular the circular dichroism spectrum, show the occurrence of a marked perturbation of both the peptide backbone and the aromatic side chains. Such a general expansion of the RNase A molecule may explain why the 2 other methionyl residues become gradually susceptible to photooxidation, so that methionine$_{29}$-methionine$_{13}$ sulfoxide-RNase A is slowly converted to the tetrasulfoxide derivative.

The preferential photooxidizability of methionine-13 with respect to methionine-30 and methionine-79 was difficult to interpret on the basis of the x-ray data. All 3 residues appear to be deeply buried in the interior of the protein molecule, being surrounded by a sheath of nonpolar side chains. The increased sensitivity to photooxidation of methionine-13 could be explained by three hypotheses: first, methionine-13 is somewhat less buried than the other 2 methionyl residues; second, the reactivity of methionine-13 is enhanced by local factors, *e.g.* presence of neighboring charges; third, the area of the RNase A molecule including methionine-13 is the first to be randomized by acetic acid.

The latter hypothesis was recently proposed by other authors

(34) as a tentative interpretation of the thermal denaturation data of RNase A.

Methionine-30 and methionine-79 remain unreactive until the final stage of the acetic acid-induced denaturation of RNase A is reached. Under these conditions, most of the native tertiary structure of the protein is destroyed so that no restrictions are imposed to the photooxidation of the methionine side chains. This is apparent from the linearity of the kinetic plot for the photooxidation of the methionyl residues in 67% acetic acid up to high percentages of conversion and from the coincidence of the rate constant for the protein-bound and free methionine. Analogously, Stark and Stein (42) found that, when RNase A is denatured by 8 M urea, each methionyl residue is alkylated at the same rate as the other 3.

The necessity for such an extensive denaturation of the RNase A molecule in order to have methionine-30 and methionine-79 available for photooxidation is to be expected on the basis of the x-ray model, as we pointed out above. Furthermore, we have recently shown (43) that in solution methionine-30 also is located very close to the ϵ-amino group of lysine-41 that is quite near to the active site of the enzyme, which is known to be hydrophobic in character.

In a previous paper (18), we demonstrated that the conversion of the uncharged thioether function of a methionyl residue to the sulfoxide in a denatured protein irreversibly stabilizes the unfolded structure, probably by decreasing the free energy of this structure with respect to the native one. Therefore, the formation of the tetrasulfoxide derivative should "freeze" the molecule of RNase A in a highly disordered situation. Actually, the conformation-dependent parameters and the circular dichroism spectra[3] determined for this product are very similar to those characteristic of RNase A after cleavage of the four disulfide bridges. In particular, the residual dissymmetry of the tyrosyl chromophores is minimal, indicating that the motility of their phenolic side chains is hindered to a very low extent. In these conditions, it is reasonable to postulate that a substantial disruption of the catalytic site of the enzyme has occurred; indeed, the tetrasulfoxide RNase A displays an extremely reduced enzymic activity.

Recently, by studying the hematoporphyrin-sensitized photooxidation of hen's egg white lysozyme (8), we succeeded in differentiating 1 buried from 1 more accessible methionyl residue. Therefore, this procedure appears to be a very valuable and reliable tool for elucidating the state of the methionyl residues in proteins. Since we recently showed (44) that another dye, crystal violet, sensitizes the selective photooxidation of cysteine to cysteic acid over a very wide range of acetic acid concentration, this technique could be utilized for determining the degree of exposure of the cysteinyl residues in polypeptide molecules. Further extension of the scope of the method may be possible by taking advantage of the conformational transitions induced by other agents, such as temperature, as preliminary experiments from other laboratories would suggest.[4] Finally, the results presented in this paper can be correlated with our recent findings about the possibility of mapping the topology of previously determined regions of protein molecules by irradiation of suitable protein-sensitizer complexes (43, 45). A concerted application of these novel photooxidative strategies and of the presently available spectroscopic techniques should yield a rather detailed description of the three-dimensional structure of proteins in solution.

[3] A. M. Tamburro, unpublished results.

[4] M. L. MacKnight and J. D. Spikes, personal communication.

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